Comprehensive Characterization of BKV Large T Antigen Epitopes to Promote the Expansion of Effectors T Lymphocytes across a Wide Range of HLA Class I and II Antigens in Prostate Cancer Patients

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Dissertation

zur

Erlangung der naturwissenschaftlichen Doktorwürde

(Dr. sc. nat.)

vorgelegt der
Mathematisch-naturwissenschaftlichen Fakultät

der

Universität Zürich

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Zurich, 2011

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Abbreviations

Aa	Amino acid
Ab	Antibody
APC	Antigen-presenting cell
Bp	Base pairs
BPH	Benign prostatic hyperplasia
BSA	Bovine serum albumin
CD	Cluster of differentiation
CEF	Cytomegalovirus/Epstein-Barr/Flu peptides-pool
CFSE	Carboxyfluorescein succinimidyl ester
Ct	Cycle threshold
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
DMSO	Dimethylsulfoxid
DNA	Deoxyribonucleic acid
dNTP	Deoxinucleotide mix
EBV	Epstein-Barr virus
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
FOXP3	Forkhead box protein 3
GM-CSF	Granulocyte macrophage-colony stimulating factor
GS	Gleason score
HCMV	Human cytomegalovirus
HD	Healthy donor
HLA	Human leukocyte antigen
HS	Human serum
IFN	Interferon
Kb	Kilo bases

Ig	Immunoglobulin
IL	Interleukin
L-Tag	Large Tumor Antigen
MACS	Magnetic cell sorting
MHC	Major histocompatibility complex
ORF	Open reading frame
P2tt830-843	Tetanus toxin universal T cell peptide
PBL	Peripheral blood leukocytes
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCa	Prostate cancer
PCR	Polymerase chain reaction
PHA	Phytohaemagglutinin
PIA	Proliferative inflammatory atrophy
PIN	Prostatic intraepithelial neoplasia
PSA	Prostate-specific antigen
PVAN	Polyomavirus-associated nephropathy
qRT-PCR	Quantitative real time polymerase chain reaction
RNA	Ribonucleic acid
RT	Reverse Transcriptase
TCR	T cell receptor
TGF	Transforming growth factor
TNF	Tumor necrosis factor
Treg	Regulatory T cell

Zusammenfassung

Prostatakrebs ist die zweithäufigste Todesursache unter den Tumorerkrankungen bei Männern. Ein aktuelles Modell für die Entstehung und Progression von Prostatakrebs muss eine Entzündung als Mitursache für die Entstehung von präneoplastischen oder neoplastischen Läsionen mitberücksichtigen. Der Polyomavirus BK (BKV) wurde schon mit Krebsvorstufen im Harntrakt assoziiert und spielt vermutlich eine wichtige Rolle in der Pathogenese des Prostatakarzinoms.

Der onkogene Effekt von BKV scheint stark mit der Aktivität des Antigens Large T (L-Tag) assoziiert zu sein, da dieses Antigen Tumorsupressorproteine zu binden und inaktivieren vermag. Dies gilt insbesondere für Proteine der Retinoblastoma Familie (pRB, p102 und p130) sowie p53. Der Mechanismus erlaubt es dem Virus, die infizierten Zellen während der produktiven Infektionsphase am Leben zu erhalten, was jedoch in nicht-permissiven Zellen zur Onkogenese führen kann.

Der Effekt des BKV L-Tag auf kritische Signalwege im humanen Zellzyklus, sowie der Nachweis von BKV und seine Expression in präneoplastischem Prostatagewebe veranlasste uns, die Rolle dieses viralen Antigens in der zellulären Immunüberwachung im Prostatakrebs zu untersuchen. Unsere Resultate unterstützen die These, nach der die CTL Immunantwort gegen virale Antigene effizient erhöht werden kann, wenn peptid-spezifische Memory T-Zellen eines seropositiven Individuums reaktiviert werden. In dieser Studie führten wir eine umfassende Analyse der CTL Antworten auf BKV large Tag Epitope in BKV-positiven Patienten mit preneoplastischen oder neoplastischen Prostataläsionen durch.

Ziel dieser Studie war es, die funktionellen Eigenschaften der BKV-spezifischen CTL Antwort in Patienten mit Prostatakrebs oder präkanzerösen Läsionen zu studieren. Als Kontrollpersonen wurden altersentsprechende Patienten mit Benigner Prostatahyperplasie sowie gesunde Kontrollpersonen jeglicher Altersgruppe untersucht. Wir identifizierten vier immunogene Peptide innerhalb des BKV L-Tag mit der Fähigkeit, eine spezifische Memory CTL Antwort zu reaktivieren. Zwei weitere Peptide, die in immunokompetenten BVK-serpositiven Probanden bekanntermassen immunogen wirken, behielten ihre erwiesene Fähigkeit, die Immunantwort auf den Virus durch Verstärkung der tolerogenen Umgebung zu regulieren. Dies bestätigte teilweise die Hypothese, dass eine ineffiziente Immunreaktionsfähigkeit auf Antigene, die spezifisch durch onkogene Viren exprimiert werde, eine Rolle in der organspezifischen Tumorgenese und der darauffolgenden neoplastischen Progression spielen kann.

Die Studie gibt einen vorläufigen Hinweis darauf, dass ein systemisches Boosting von Prostatakarzinompatienten mit immunogenen Domänen des BKV L-Tag eine T-Zell Immunantwort zugunsten einer effektiven proinflammatorischen Immunaktivität bewirkt. Dies

hebt die Immuntoleranz auf, die teilweise durch das BKV L-Tag induzierte regulatorische Profil gesteuert wird.

Abstract

Prostate cancer (PCa) is the third leading cause of cancer death in men. A contemporary model for prostate cancer induction and progression should include the potential contribution of inflammation to the development of preneoplastic or neoplastic lesions. The Human polyomavirus BK (BKV) has been associated to pre-early stages of cancer in urinary tract and it is postulated to play an important role in the pathogenesis of prostate cancer.

The BKV oncogenic effect appears to be highly associated to the activity of the Large T antigen (L-Tag) because of its capability to bind and inactivate products of tumor suppressor genes, specifically tumor suppressor proteins of the retinoblastoma family (pRb, p107, and p130) and p53. This mechanism is used by the virus to keep the infected cells alive during the productive infection but in non-permissive cells it may lead to oncogenesis.

The involvement of BKV L-Tag in the alteration of critical pathways of the human cell cycle together with its detection and expression in preneoplastic prostate tissues prompt us to investigate the role of this viral antigen as target of cellular immune surveillance in prostate cancer. Our data support the concept that CTL immune responses against viral antigens can be effectively expanded by reactivating peptide-specific memory T cells from seropositive subjects. In this study we propose a comprehensive analysis of CTL response against BKV large T antigen-related epitopes in BKV-experienced patients bearing prostate preneoplastic or neoplastic lesions irrespective of their HLA restrictions.

The specific aim of this study was to fully characterize the functional features of BKV specific CTL responses in patients bearing prostate cancer or precancerous lesions, as compared to gender-matched controls (age-matched benign prostatic hyperplasia patients and non age-matched healthy donors). To this end we identified four immunogenic peptides within BKV L-Tag able to reactivate a specific memory CTL responses when challenged for their *ex vivo* and *in vitro* capability to recall CD4+ and CD8+ T cells. In addition, two peptides proven to be immunogenic in immunocompetent BKV seropositive subjects, maintained their known potential to regulate the immune response to the virus through the enhancement of a tolerogenic environment. This partially confirmed the hypothesis that an inefficient immune responsiveness to antigens specifically expressed by oncogenic viruses might play a role in organ specific tumorigenesis and subsequent neoplastic progression.

This study gives preliminary evidence that a systemic boosting of PCa patients using immunogenic domains within BKV L-Tag would implement a T cell immune response in favor of an effective pro-inflammatory immune activity. This would lead to “the breaking of the immune tolerance” which is partially governed by BKV L-Tag in PCa.

1. Introduction

1.1. The Prostate

Prostate is an accessory reproductive gland of the male reproductive system. It surrounds the urethra and lies with its superior surface below the urinary bladder and with its inferior surface above the pelvic musculofacial floor. The dimensions of a healthy human prostate are similar to a walnut, with a weight around 20g. The prostate counts four distinct glandular regions, which represent the zonal anatomy classification. The peripheral zone (PZ) composes the 70% of the prostate volume, whereas the central zone (CZ) and the transitional zone (TZ) constitute the 20% and the 5%, respectively. The fourth zone is the fibro-muscular zone (or stroma), which accounts for the 5% of the prostate weight. In pathology, this classification is often used. Mostly cancers develop in the PZ, while benign hyperplasia does in the TZ of the prostate gland (McNeal, Redwine et al. 1988). The prostate is composed of fibromuscular stroma and of 30 to 50 glands surrounded by a thin layer of connective tissue, that drain into the prostatic urethra (McNeal 1972) (Blacklock 1974) (McNeal, Redwine et al. 1988).

The main function of the prostate is to secrete a milky, alkaline fluid into the urethra during ejaculation. This fluid constitutes 25-30% of the volume of the semen. Its function, exerted through its alkalinity, is to nourish, to protect and to increase the lifespan of sperm. The prostate also help expel semen during ejaculation. Another important function is controlling the flow of urine during ejaculation.

The size and the function of prostate are regulated by hormones secreted by either the hypothalamic pituitary-testicular axis or adrenal gland. Testosterone, the main male hormone driving to anabolic and androgenic effects in men, is of great importance for the growth and maintenance of prostate gland. Products of testosterone catabolism targets specific DNA sequences leading to activation of cell functions, including cell-growth and proliferation (Carlson and Katzenellenbogen 1990; Deslypere, Young et al. 1992) (Russell and Wilson 1994).

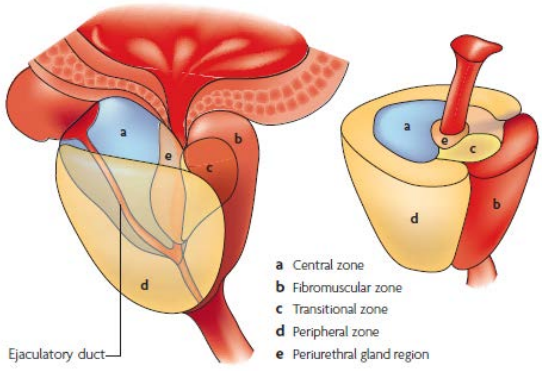

Figure I - 1. The anatomy of prostate
(From (De Marzo, Platz et al. 2007)

1.2. Prostate cancer

1.2.1. Epidemiology

Prostate cancer (PCa) represents the first leading cause of cancer morbidity and the third of cancer death in men in developed countries with a worldwide incidence rate accounting for 14% of total newly diagnosed cases and a worldwide total cancer mortality rate of 6% (Gonzalgo and Isaacs 2003) (Cooperberg, Moul et al. 2005; Jemal, Bray et al. 2011). The most affected are African American men in the United States (SEER Program (National Cancer Institute (U.S.)), National Center for Health Statistics (U.S.) et al. 1993). Differences among populations might be caused by a combination of underlying aspects such as genetic susceptibility and differences in health care. Moreover, the importance of dietary, socioeconomic and environmental factors is illustrated by the increasing risk of PCa in Asian immigrants in the US (Whittemore, Kolonel et al. 1995). Genetic alterations probably also contribute to the huge geographical variation of PCa incidence.

The PCa increasing incidence in industrialized countries might be due to the growing awareness of this disease and the more intense, refined and widespread diagnostic procedures, such as Prostate Specific Antigen (PSA) test and random biopsies (Potosky, Miller et al. 1995). In contrast, PCa mortality is declining owing to the improvement, as plausible explanation, in the management of this disease. Changes in PCa treatment include refinements in the techniques of radical prostatectomy and radiation therapy for early stage disease. It is likely that the more widespread use of these treatments is associated with increased numbers of men presenting with early stage disease, most of which probably detected through PSA screening.

1.2.2. Etiology and risk factors

Despite several research efforts, the cause of PCa remains unclear. It has been assumed that PCa has a multi factorial origin with environmental as well as genetic factors. To summarize, risk factors associated with increased incidence rate are: i) advanced age with an average age of 72 years at first diagnosis due to a longer life expectancy (the tumor is almost rare in 40-45 years old men); ii) family history of PCa (9% of PCa, genetically passed on in an autosomal dominant fashion); iii) race (African Americans develop PCa 50% more frequently than their Caucasian counterparts at the same age); iv) hormones activity (androgens and insulin-like growth factor); v) lower rate of daily dietary fibers and higher rate

of fat (Littrup 1997; Brawley 1998; Brawley, Knopf et al. 1998).

Aging is the most relevant risk factor (Abate-Shen and Shen 2000). The mean age of patients diagnosed for PCa is 72-74 years, with about 85% after the age of 65. PCa is diagnosed rarely in people aged younger than 50 years (Sakr, Haas et al. 1993). Results of autopsy studies have reported that most men aged older than 85 years have histological PCa. Consequently, it has been suggested that most men would contract PCa if they live long enough (Sakr, Wheeler et al. 1996).

The geographical incidence of PCa induced researchers to investigate its possible association with the western lifestyle and in particular diet that includes a high intake of fat, meat, and dairy products (Howell 1974). The results of these studies are mostly inconsistent, reflecting the complex multifactorial nature of this disease (Hayes, Ziegler et al. 1999) (Whittemore, Kolonel et al. 1995). However, several studies on animal model showed an over expression of AMACR (α-methyl-CoA remarcase) in PCa (Luo, Zha et al. 2002). This gene plays a key role in the metabolism of branched fatty acids, which is a potential source of carcinogenic oxidative damage. Because beef and dairy products are major sources of dietary branched fatty acids, up-regulation of AMACR might support the hypothesis of an association between “Western” diet and PCa. In addition, the western lifestyle has contributed to marked increase in the percentage of overweight and obese men over the last decades. Consequently, obesity has been considered a possible risk factor of PCa as well (MacInnis and English 2006). Results on this field are very contradictory, nevertheless some studies have shown an association between increased Body Mass Index (BMI) and risk of PCa mortality, suggesting that obesity might play a more important role in the progression of the disease than in its initiation (Rodriguez, Patel et al. 2001) (Rodriguez, McCullough et al. 2003). Inflammation, arisen from exposure to environmental factors such as infectious agents and hormonal imbalances, is another risk factor that is likely involved in PCa development (De Marzo, Platz et al. 2007). Many others risk factors have been hypothesized and investigated, specifically the influence of occupation, sexual and physical activity and social factors (Bostwick, Burke et al. 2004).

1.2.3. Cytogenetic

Recurrent genetic aberrations in PCa have been documented over the last decade. The most frequent aberrations are losses in chromosomes 5q, 6q, 8p, 10q, 13q, 16q, 17p, and 18q, and gains in 7p/q, 8q, 9p, and Xq (Alers, Rochat et al. 2000). Target genes for these aberrations remain undefined mainly because of the heterogeneity of the disease (Amanatullah, Reutens et al. 2000). However, two genes for as many two chromosomal

aberrations have been identified: the androgen receptor (AR) at gene Xq12 and the TMPRSS2-ERG gene at 21q. The latter is a chromosomal translocation where the transcription factor related genes (ERG) is juxtaposed to androgen regulated type-2 transmembrane serine protease (TMPRSS2) gene. It represents a recurrent gene fusion (TMPRSS2-EGR) occurring in up to 50% of clinically localized PCa (Tomlins, Rhodes et al. 2005) (Tomlins, Mehra et al. 2006). Others putative target genes include: prostate-specific homeobox gene NKX3-1 - its loss expression correlate with tumor progression - (Bowen, Bubendorf et al. 2000) and Glutathione S-transferase P1 (GSTP1); its promoter-hypermethylation is the most commonly described epigenetic alteration in PCa (DeMarzo, Nelson et al. 2003).The identification of target genes correlating to chromosomal aberrations in PCa will provide new prognostic markers and therapeutic targets for future drug development.

1.2.4. Pathology of prostate cancer

Prostate adenocarcinoma is the most common histological type among prostate cancers accounting for more than 90% of the cases (Cookson 2001). It usually arises from the active glandular epithelium along the periphery of the gland. PCa is an organ confined low risk cancer with low aggressive potential (usually, only 1 out of 6 PCa are lethal cancers

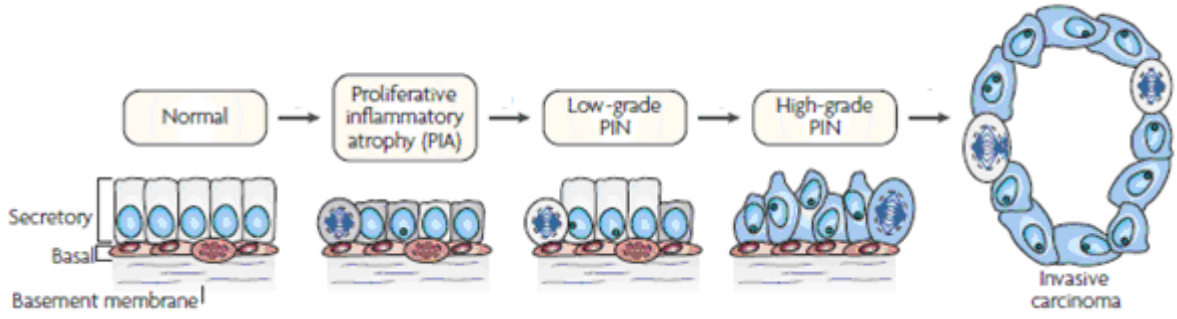

Figure I - 2. Cellular model of early prostate neoplasia progression
(From (De Marzo, Platz et al. 2007)

- Gleason score 8 to 10 at first diagnosis; 50% survival rate at 5 years). It has been reported that PCa at prostate intraepithelial neoplasia (PIN) stage (tumor size >0.5 cm^3; Gleason score 2 to 4) progress to the level of overt cancer (tumor size of 3-4 cm^3) in about 10 years, and metastasize (preferentially in bone and brain) in about 15 years (Pound, Partin et al. 1999). Improvement of pre-emptive screenings (PSA test) has reduced the frequency of localized invasive cancers in patients at the time of first diagnosis with a

dramatic shift toward a “watchful waiting” treatment of choice. The latter has dramatically reduced source of tumor samples and limited the development of compelling research strategies, also owing to the intrinsic properties of PCa that render this tumor a difficult target (Schlomm, Erbersdobler et al. 2007). It has thus prompted researchers to focus on the identification of early stages of the tumor to better understand the mechanism responsible for PCa onset and progression.

Despite the high prevalence and importance of PCa, the pathogenesis mechanisms underlying its development and progression remain poorly understood. It is supposed that transition from benign prostate over pre-neoplastic lesions to PCa is a continuous process (Bostwick and Brawer 1987). Several potential precursor lesions of PCa have been described, specifically low grade prostatic epithelial neoplasia, atrophy and atypical adenomatous hyperplasia.

The proliferative inflammatory atrophy (PIA) of the prostate has recently gained relevance as potential precursor of prostatic intraepithelial neoplasia (PIN) and overt PCa (De Marzo, Platz et al. 2007), in particular owing to the prevalence of PIA in the peripheral zone of the organ, where usually histological transitions between PIA and PIN occur (De Marzo, Marchi et al. 1999; Putzi and De Marzo 2000) (Figure I - 2). However the most convincing data are those reporting high grade prostatic intraepithelial neoplasia (HGPIN) as precursor lesion of PCa (Montironi, Mazzucchelli et al. 2007).

1.2.4.1. Proliferative inflammatory atrophy (PIA)

Inflammation is a frequent finding in PCa. Many lesions containing inflammation are associated with either diffuse or focal epithelial atrophy. While most focal prostatic atrophy lesions have been considered to be quiescent, cells in some atrophy lesions appear proliferative. The term proliferative inflammatory atrophy (PIA) has been coined to define these proliferative glandular epithelial cells (De Marzo, Marchi et al. 1999). PIA lesions seem to arise in response to inflammatory injury caused by a variety of potential agents, among which infection (chronic bacterial infection, fungi, mycobacteria and latent viruses), cell injury (owing to exposure to chemical and physical trauma from urine reflux), hormonal variations and dietary factors.

As documented by De Marzo et al., PIA may also emerge as a consequence of epithelial damage caused by oxidative stress. Oxygen radicals can directly damage DNA resulting in the accumulation of genomic aberrations. Moreover, modifications of specific genes may also be involved with elevated oxidative stress. For instance, Glutathione *S*-transferase P1 (GSTP1) is a detoxification enzyme that helps to catalyze conjugation

reactions between potentially damaging oxidants and glutathione. GSTP1 is hypermethylated in more than 90% of PCa, this inactivation might leave cells vulnerable to oxidative DNA damage (Nakayama, Bennett et al. 2003).

Many of the molecular and genetic changes seen in PIA are also documented in HGPIN and PCa. Morphological studies observed merging of focal atrophy with HGPIN and close relation to early carcinoma (De Marzo, DeWeese et al. 2004). In addition, PIA, HGPIN and PCa share similar locations in the prostate zones (McNeal 1988). As consequence of all these findings it has been postulated that PIA may represent precursor lesion to prostatic intraepithelial neoplasia and, therefore, prostatic carcinoma (De Marzo, Marchi et al. 1999).

1.2.4.2. Prostatic intraepithelial neoplasia (PIN)

The term prostatic intraepithelial neoplasia (PIN) was introduced by Bostwick and Braver in 1987. PIN is defined as an intra-acinar epithelial proliferation with significant nuclear atypia in the secretory cells (Bostwick 1988). The 3-tier grading system has been accepted to discriminate between low grade (LG) and high grade (HG) PIN. Low-grade (LG) PIN included grade 1 and high-grade (HG) PIN included grade 2 and 3. HGPIN may display a spectrum of 4 architectural patterns, which are: tufting, micropapillary, cribriform and flat.

HGPIN and PCa are both multifocal and share similar locations in the prostate zones (Montironi, Mazzucchelli et al. 2000). HGPIN is strongly predictive of the presence of carcinoma; consequently the identification of HGPIN in biopsy has important clinical implications. PCa and HGPIN share several cytological features such as an increased proliferation and apoptosis (Montironi, Galluzzi et al. 1993), they are morphologically similar (Vis and Van Der Kwast 2001) and they have in common some molecular and genetic alterations (Qian, Jenkins et al. 1995). For all these reasons HGPIN is considered to be the most likely precursor of invasive PCa.

1.2.5. Prognostic and predictive factors of PCa

Prognostic factors characterize patients' situations or conditions that can be used to estimate the chance of recovery from a disease or the chance of the disease recurring.

Prediction of prognosis is one of the greatest challenges in tumor pathology (Burke, Bostwick et al. 2005). Despite all the efforts done to introduce new molecular biomarkers in the prediction of cancer prognosis, histopathological grade remains the most predictor factor for neoplastic diseases.

Prognostic factors can be classified in three categories according to the College of

American Pathologist (CAP) (Bostwick, Grignon et al. 2000). The first category homes markers that are supported by robust data in the literature and are conventionally used in the management of patients. Considering PCa, preoperative PSA and histologic grade (Gleason score) belong to this category. These markers are used in clinical practice in the form of nomograms for prediction of tumor progression (Kattan, Wheeler et al. 1999). The second category includes markers that have been extensively studied at either biological or clinical level but with few clinical outcome data. In PCa, tumor volume and DNA ploidy belong to this category. The third category groups all markers that do not meet the criteria of category I or II. Examples are molecular markers of proliferation and angiogenesis, tumor suppressor genes, apoptotic genes and oncogenes, and more.

Despite extensive research efforts, few biomarkers for PCa prognosis have been introduced to date in clinical practice. The clinical nomograms based on Gleason grade, tumor stage and serum level of PSA are still the best predictors of PCa outcome (Fiorentino, Capizzi et al.). However, the biotechnological advancements achieved in the last decade represent a remarkable source for new prognostic and predictive tissue and serum molecular biomarkers.

1.2.5.1. Gleason score

Donald F. Gleason worked on his grading system from 1960 to 1974. It has been developed by large patient population with long follow-up (Bailar, Mellinger et al. 1966) (Gleason 1966) (Gleason and Mellinger 1974) (Gleason 1988) (Gleason 1992).

The Gleason score (GS) is today the grading system for PCa officially recommended by the World Health Organization (Eble JN 2004). PCa is graded according to Gleason system on specimen coming from needle biopsies, transurethral prostate resection (TUR-P) and radical prostatectomy (Brinker, Potter et al. 1999) (Brinker, Ross et al. 1999) (Amin, Boccon-Gibod et al. 2005) (Montironi, Lopez-Beltran et al. 2001) (Epstein, Srigley et al. 2007) (Srigley, Amin et al. 2006).

The Gleason score grading is based on the analysis of the glandular architecture of prostate tissue sections and does not take into consideration cytological features. The GS grading system accounts for five distinct patterns that prostate tumor cells tend to go through as they change from normal cells. The scale runs from 1 to 5, where 1 represents cells that are very nearly normal and 5 represents cells that don't look much like prostate cells at all (Figure I - 3). The pattern determination is performed by visual estimation of tissue specimen under the microscope. The primary and secondary pattern (i.e. the most prevalent and the second most prevalent pattern) are added to obtain the GS (Gleason

pattern 1+ Gleason pattern 2). Obviously the score (sum) can range from 1 to 10. If the specimen has only one pattern, the GS is obtained by doubling that pattern.

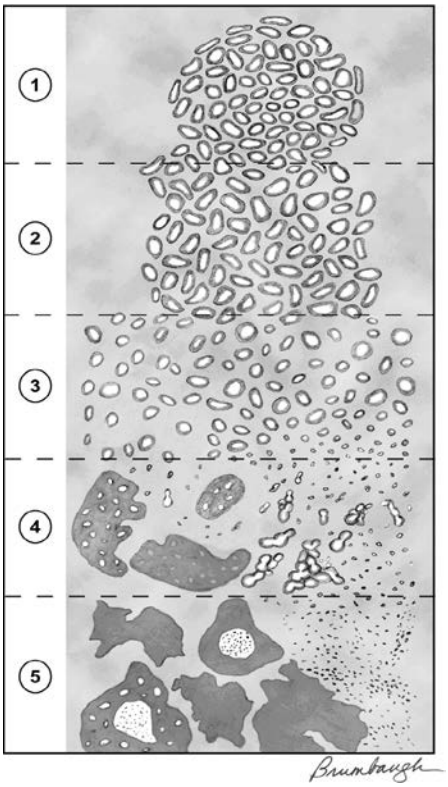

Figure I - 3. Modified Gleason System (From (Epstein 2010)

The strength of the Gleason score system lies in the fact that it is the most powerful predictor of patient outcome. It prevalently correlates with multiple other parameters related to prognosis, such as age (Draisma, Postma et al. 2006), serum PSA (Horninger, Rogatsch et al. 1999), clinical stage (Ramos, Carvahal et al. 1999) and pathological stage (Helpap and Egevad 2006). Moreover, it validity as independent effective prognostic factor for both prediction of natural PCa history and recurrence risk assessment after radical prostatectomy has been well documented (Berney, Fisher et al. 2007) (Han, Partin et al. 2003).

The Gleason score system presents some weakness as well, mainly because of a pronounced clustering in the mid range pattern. A modification of the scoring system has been a long time debated. In 2005 the International Society of Urological Pathology (ISUP) has solved this issue providing new recommendations for a modified Gleason system (Epstein, Allsbrook et al. 2005). Epstein JI has recently reviewed the modification introduced to Gleason score (Epstein). A schematic drawing of a modified Gleason system is depicted in Figure I - 3. The modified diagram shows the changes, which mainly involve pattern 3 and 4.

1.2.5.2. Biomarkers in prostate cancer

By definition, biomarkers are characteristics that are objectively measured and evaluated as indicators of normal biologic processes, pathogenic processes, or pharmacologic responses to therapeutic interventions. In prostate cancer, they should be specific and sensitive independent agents detectable either in blood or urine to discriminate between indolent and clinically significant cancers.

So far, the relevant marker for diagnosis and therapeutic decision in PCa is the Prostate specific antigen (PSA). PSA is an enzyme produced by the cell of prostate gland to liquefy the semen. It can be detected in blood of patients (PSA cutoff≤4ng/ml). Together with

the digital rectal examination (DRE) leads to patients' therapeutic decision and serum PSA analysis contributes to patient stratification into different prognostic categories (Partin, Mangold et al. 2001). It is currently in use as marker for "active surveillance" (van den Berg, Roemeling et al. 2007) (Hugosson, Carlsson et al. 2010) and is a perfect indicator for PCa recurrence (0.02ng/ml on three consecutive blood tests) (Schmidt 2010). Although its effectiveness as prognostic factor PSA is associated with several limitations. The enzyme is organ but not cancer specific and can be influenced by several factors like prostate volume, patients' age, treatment for benign prostatic hyperplasia (5-alpha-reductase-inhibitor). Indeed, relationship between serum PSA and patient outcome is influenced by coexisting prostatic hyperplasia (Beduschi and Oesterling 1997), patient age (Richardson and Oesterling 1997) and, eventually, Gleason grade (Partin, Carter et al. 1990).

To date, molecular staging of PCa specimens was the only procedure available for PCa diagnosis, prognosis, and therapeutic decision. However, only a minority of studies carried out on PCa tissue specimens identified prognostic relevant markers, such as p53, Ki-67, Bcl-2 and AR, that correlated with clinical endpoints (Schlomm, Erbersdobler et al. 2007). In addition, tissue specimens presuppose surgical interventions on patients as prostate biopsies or prostatectomy.

To invest on PCa diagnosis and prognosis "before biopsies", several efforts have been devoting on easy screening through the identification of specific biomarkers in human serum or urine, either at gene or protein level. Among them, the PCA3 test is the relevant one. The test screens the release after prostate massage/DRE of a non-coding mRNA from DD3 gene in urine of patients at risk of PCa. This non-functional gene is cancer specific, correlates with PCa positive biopsies and its production is independent of prostate volume and patients' age. However, some weaknesses such as negative results in patients with PIN, correlation with Gleason grade and cancer volume render it not suitable as primary diagnostic test (Roobol, Schroder et al. 2010). The combination of two or more markers would enhance the sensitivity and specificity of screening tests. Indeed, a marker panel composed by PCA3, AMACR and the TMPRSS2-EGR gene, due to chromosomal translocation at 21q22.3, as multiplex gene test for PCa patients' urine is currently in use.

Targeting canonical markers and gene rearrangements might become an important innovative approach in solid tumors management. Taking advantage of new highly sensitive methodologies (DNA array, qRT-PCR, FISH, proteomics) would implement the number of predictive markers to test in universal and affordable easy screenings (Provenzano 2012).

1.3. Prostate cancer and viral association

1.3.1. Human polyomavirus BK

1.3.1.1. Etiology, epidemiology and clinical manifestation

Human polyomavirus BK is a dsDNA virus belonging to the taxonomic family Polyomavirinae that comprises 13 distinct viruses with a common ancestor that exhibits a limited host range of host species to infect (Cole 1996). Human polyomaviruses are BKV, JCV, and the newly discovered polyomaviruses WUV (Washington University polyomavirus) (Gaynor, Nissen et al. 2007), KIV (Karolinska Institute polyomavirus) (Allander, Andreasson et al. 2007), and MCV (Merkel cell carcinoma polyomavirus or MCPyV) (Feng, Shuda et al. 2008). They share approximately a 70-75% genome homology.

Human Polyomavirus BK has been first identified in 1979 isolated from urine of a Sudanese patient (with initial B.K.) suffering from urethral stenosis after kidney transplantation (Gardner, Field et al. 1971). The BK virus is ubiquitous in the human population worldwide (Padgett and Walker 1976) establishing a life-long latent infection, except in some populations of Brazil, Paraguay and Malaysia (Brown, Tsai et al. 1975). Serological evidence indicates that nearly 90% of individuals are infected by early childhood, although a decrease in this rate (70-80%) during the human lifespan is reported (Taguchi, Kajioka et al. 1982).

After primary infection the virus disseminates and establishes a latent infection in renal tubular epithelial cells and urothelial cell layers, the latter representing the principal site of viral latency (Imperiale 2000; Hirsch and Steiger 2003). It is also possible to detect BKV in liver, stomach, lung, lymph nodes (Israel, Martin et al. 1978; Pater 1980) and lymphocytes (Dorries, Vogel et al. 1994; Degener, Pietropaolo et al. 1997). The presence of BKV in tonsillar tissue (Goudsmit, Wertheim-van Dillen et al. 1982) suggests for respiratory tract as the initial site of viral replication. However, primary infection is thought to be asymptomatic although no specific upper respiratory symptoms have been noted in some individuals (Shah 1996). The route of transmission may thus occur via respiratory secretions or exposure to urine (Major 2001). Spontaneous reactivations and low-level replication with shedding into urines is observed in 5-20% of healthy individuals (Hirsch 2005).

Usually polyomaviruses cause persistent subclinical infections in humans and BKV infection rarely leads to clinical manifestation. Mild pyrexia, malaise, vomiting, respiratory illness, pericarditis and hepatic dysfunctions have been reported with primary infection

(Fioriti, Videtta et al. 2005). Reactivation in immune competent individuals with intermittent low-level urinary replication (BKV loads of $\leq 10^6$/ml) and urinary viral shedding has been detected in 5% of infected subjects (Shah, Daniel et al. 1997). However, when the immune system is compromised, as following solid organ and bone marrow transplantation, HIV infection, chemotherapy or pharmacologic immune suppression, rate and level of BKV replication increase and may lead to organ disease (Gardner, MacKenzie et al. 1984; Leung, Suen et al. 2001; Hirsch, Knowles et al. 2002).

Polyomavirus-associated nephropathy (PVAN) is the most challenging infectious in immune compromised kidney transplanted patients that leads to renal allograft dysfunction and graft loss (Hirsch and Steiger 2003; Hirsch 2005). BKV viral shedding in the urine is a useful test to exclude PVAN in kidney recipients (Comoli, Hirsch et al. 2008), although distinguishing between asymptomatic viral shedding in the urine of immune competent subjects (0.3%) and BKV disease (10%-45%) caused by viral reactivation (PVAN, ureteric stenosis, hemorrhagic cystitis) remains difficult (Fioriti, Videtta et al. 2005).

Recent investigations have pointed out the role of viral non-coding control region (NCCR) variants in viral host cell permissiveness, rate of viral replication and type of disease diagnosed in BKV infected subjects (Gosert, Rinaldo et al. 2008).

1.3.1.2. Viral structure and mechanisms of viral replication

Human polyomavirus BK (BKV) is an on average 5 kilo base pairs (kbp) closed circular double-stranded (ds)DNA virus (5,153 bp for Dunlop strain (Dhar, Lai et al. 1978; Seif, Khoury et al. 1979) (Figure I- 1); 4,963 bp for strain MM (Tavis, Walker et al. 1989); 5,098 bp for strain AS (Yoshiike 1986)). BKV DNA is packaged as circular mini-chromosome in a complex with histone proteins (H2A, H2B, H3 and H4) and enclosed by a non-enveloped icosahedral capsid composed of three viral proteins (VP1, VP2, and VP3) (Eash, Manley et al. 2006).

The genome of BKV consists of the hypervariable non-coding control region (NCCR) located between the genetically conserved coding regions for early genes and late genes. The NCCR drives viral gene expression. It contains, beyond the relevant cellular transcription factor binding sites, the origin for viral DNA replication (*ori*), promoter and enhancer for transcription of early and late genes and T antigens (Tags) binding sites (Cole 2001; Moens 2001). The coding regions for early genes (L-Tag and small tumor antigen, s-tag) are transcribed in a counter-clockwise direction while coding regions for late genes (agnoprotein, major structural protein VP1 and the two minor structural proteins VP2 and

VP3) in a clockwise direction (Lednicky and Butel 1999). All proteins from each of the two coding regions are derived from a common precursor mRNA by alternate splicing. In particular, the early region is the first part of the viral genome to be transcribed and translated since encodes the two viral lifecycle regulatory L-Tag and s-tag. L-Tag has been identified early on as a key regulatory molecule interacting with infected cell cycle (Fanning and Knippers 1992). Differently, the role of s-tag in the lifecycle of polyomavirus is unknown and it has been given an ancillary role for L-Tag activity, such as increasing virus yield in permissive cell infection (Rundell and Parakati 2001).

The infection cycle of BKV is divided into two phases: early and late. The first phase comprises viral entry and DNA replication while the second phase consists on assembly of new virions and release of viral progeny. Viral entry is governed by caveolae-mediated endocytosis (Eash, Querbes et al. 2004) that allows viruses to reach the nucleus of infected cells where their replication takes place (Drachenberg, Papadimitriou et al. 2003). Upon early viral gene transcription, L-Tag accumulation initiates viral DNA replication in the nucleus of infected cells by binding the NCCR *ori* region, promoting unwinding of DNA and recruiting the host cellular DNA polymerase and replication proteins (Imperiale 2007). Late genes expression encoding for structural proteins (VP1, VP2, and VP3) as well as expression of the regulatory agnoprotein, will follow. Viral assembly is achieved in the nucleus. The three viral structural proteins (VP1, VP2, and VP3) assemble with the

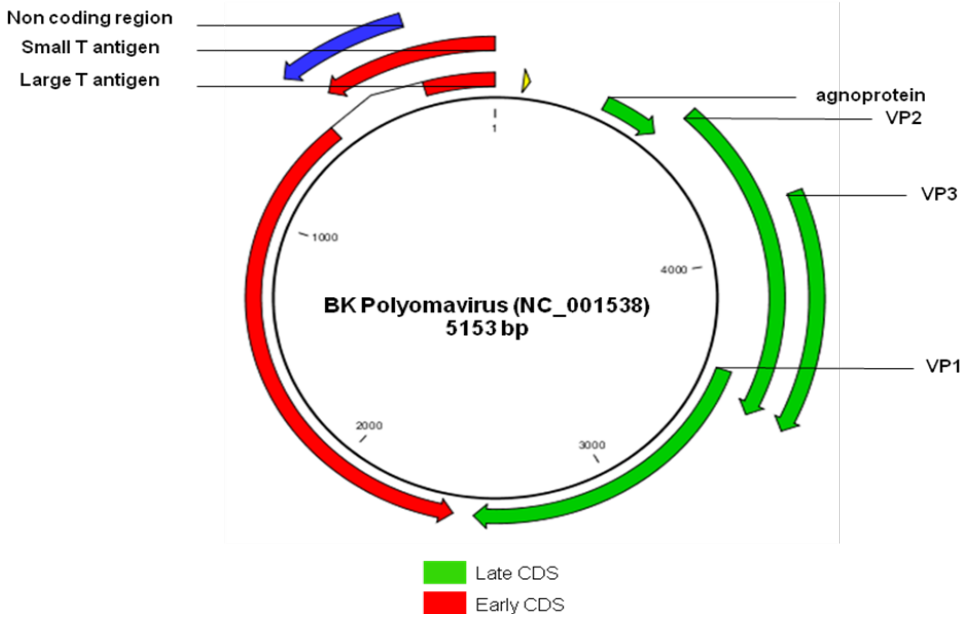

Figure I - 4. Schematic organization of the human BK polyomavirus genome

replicated viral DNA to form virions that are released upon cell lysis (White and Khalili 2004).

In the host species permissive cells, polyomavirus BK spreads by lytic infection. However, in non-permissive cells, in the context of an abortive infection, it leads to cellular transformation (White and Khalili 2004; White and Khalili 2005), although in the presence of activated oncogenes, such as c-Harvey-ras (Pagnani, Corallini et al. 1988), adenovirus E1A, c-rasA, c-myc (Corallini 2001). In particular, the two BKV early gene products L-Tag and s-tag play a relevant role in cell immortalization and neoplastic transformation (Imperiale 2000; Imperiale 2001).

As seen above, the role of L-Tag in the viral lifecycle is relevant for productive infections while the role of s-tag in this context is less unclear (Shenk, Carbon et al. 1976). Differently, L-Tag and s-tag cooperate in the transformation of infected cells (Martin, Setlow et al. 1979; Martin, Setlow et al. 1979; Porras, Bennett et al. 1996; Yu, Boyapati et al. 2001). L-Tag is a nuclear phosphoprotein that promotes cellular transformation by interacting with products of tumor suppressor genes: retinoblastoma (Rb) gene product (pRb), Rb family members p107 (Ewen, Xing et al. 1991) and p130 (pRb2) (Hannon, Demetrick et al. 1993), and wild-type p53 (Bocchetta, Eliasz et al. 2008), thus interfering with physiological check points of cell cycle (Tognon, Corallini et al. 2003; Ahuja, Saenz-Robles et al. 2005). L-Tag-tumor suppressor gene products (thereafter referred as pRbs) binding modulates cellular signaling pathways that stimulate progression of infected cells into S phase in order to optimize the environment for viral replication (Fioriti, Videtta et al. 2005; Provenzano, Eliceiri et al. 2006). Therefore, this mechanism is used by the virus to keep infected cells alive during productive infection but in the context of non permissive cells it turns out into neoplastic transformation (Imperiale 2000).

In details, L-Tag binds to pRb, p107, p130 competing with the transcription factors E2Fs (E2F-1 to E2F-8) for the re-phosphorylation of pRb, thereby interfering with E2Fs control activity and inducing infected cells to enter the cell cycle (S phase). In fact, pRbs-E2Fs binding (E2F1, 2, 3a and 3b for pRb; E2F4 and E2F5 for p107 and p130) (Weinberg 1995; Weinberg 1996) maintains pRb in a hypophosphorylated form in G0 and its phosphorylation during G1 to S progression is due to actions of mitogenic signals through cyclin-dependent kinases (Cobrinik 2005; Provenzano, Eliceiri et al. 2006). However, L-Tag-pRb, p107, and p130 interactions are limited due to the low expressed levels of the BKV regulatory protein. Nevertheless, L-Tag alters phosphorylation patterns of pRbs members inducing serum-independent growth of infected cells (Harris, Chang et al. 1998; Harris, Christensen et al. 1998). Relevant is also the notion that polyomavirus L-Tag-pRbs binding is not stable during cell cycle phases (Ludlow, Shon et al. 1990).

Sequestration of p53 by L-Tag in the cytoplasm of infected cells represents the main step for their malignant transformation. The latter requires p53 inactivation to prevent: a) cell cycle arrest; b) DNA repair; c) cell apoptosis (Pipas and Levine 2001). The tumor suppressor p53 is able to mediate cell cycle arrest by inducing p21 expression, a cyclin-dependent kinase inhibitor (Xiong, Hannon et al. 1993) that in turn causes cell arrest. It also drives to DNA repair in genetic damaged cells thus avoiding propagation of genetic mutations or aberrations to daughters' cells (Bocchetta, Eliasz et al. 2008). L-Tag-p53 binding stabilizes the protein but inactivates its tumor suppressor function, thus mimicking the phenotypic effects caused by mutations in the p53 gene (Figure I - 5). The coexistence of BKV L-Tag and wild-type p53 in the cytoplasm of infected cells (Das, Shah et al. 2004) is a key point for supporting a BKV intervention in the genesis of human cancers (Das, Wojno et al. 2008).

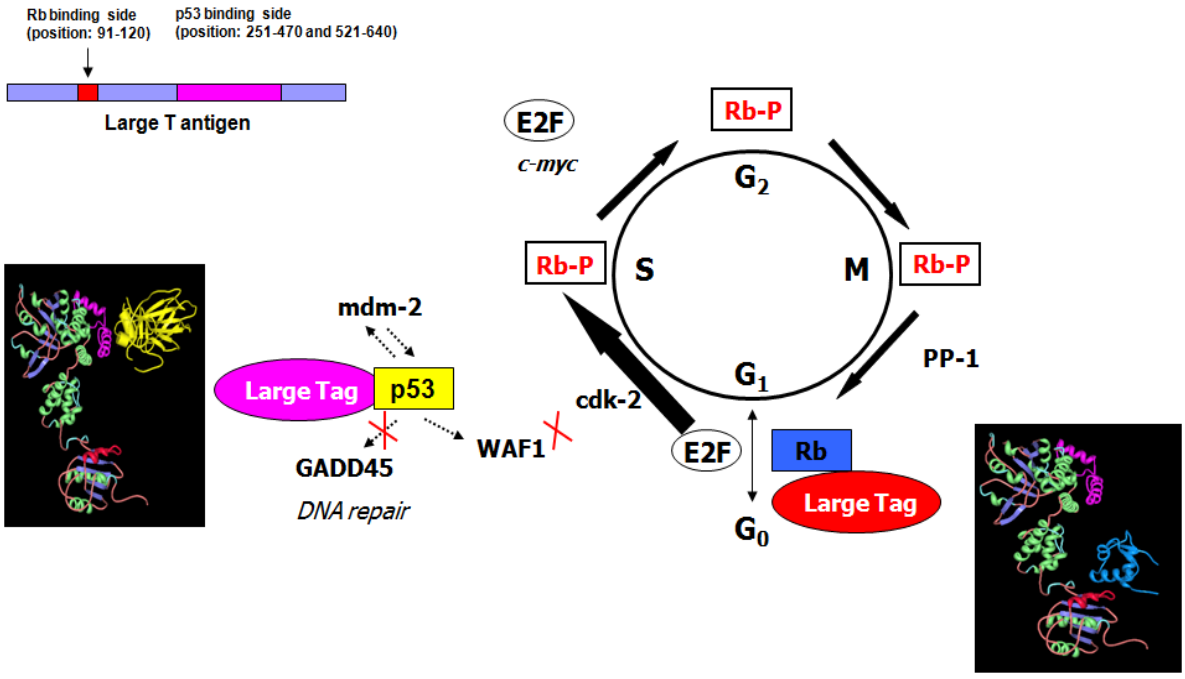

Figure I - 5. Interaction of BKV L-Tag with pRbs and p53

1.3.2. Association of BKV with human cancers

1.3.2.1. Background

The role of human polyomavirus in human cancers is still debated. Recent investigations have associated polyomavirus with the outgrowth of specific cancer types including colorectal cancer (Enam, Del Valle et al. 2002; Casini, Borgese et al. 2005), glioblastomas (Tognon, Casalone et al. 1996; Del Valle, Delbue et al. 2002), mesotheliomas (Carbone, Rizzo et al. 1997), prostate cancer (Das, Shah et al. 2004), lymphomas (Engels, Rollison et al. 2005; Rollison, Engels et al. 2006). Complete viral polyomavirus DNA genome or fragments containing the early region are able to transform embryonic fibroblasts and cells cultured from kidney or brain of mouse, rat, hamster, rabbit and monkey (Corallini 2001; Tognon, Corallini et al. 2003). It has rendered polyomavirus as prototypes of DNA tumor virus well amenable to studies in experimental models. However, the possible role for polyomavirus BK in human cancers is still controversial.

In human, transformation of human cells by BKV is not efficient and often abortive (Tognon, Corallini et al. 2003) although BKV-infected or transfected cells show morphological alterations and increased lifespan (Grossi, Caputo et al. 1982). A complete transformed phenotype could be seen in human infected cells when they are *in vitro* transfected with recombinant DNA containing BKV early region genes (L-Tag) and oncogenes, such as c-rasA (pBK/c-rasA) or c-myc (pBK/c-myc), as seen in human embryo fibroblasts (Pater and Pater 1986) and adenovirus E1A (pBK/12-E1A) in human embryonic kidney (HEK) cells (Vasavada, Eager et al. 1986).

So far, several scientific works examining the presence of BKV in human cancers using different techniques as polymerase chain reaction (PCR), immunohistochemistry (IHC), *in situ* hybridization (ISH) are available in the literature. Among them, some reported either BKV DNA, or viral RNA or proteins presence in the tumor specimens tested (Pagnani, Corallini et al. 1988; Imperiale 2000; Tognon, Corallini et al. 2003; White and Khalili 2004). In contrast, some other reports found no evidence for BKV in human tumors (Weggen, Bayer et al. 2000; Knoll, Stoehr et al. 2003; Bergh, Marklund et al. 2007; Lau, Lacey et al. 2007; Rollison, Sexton et al. 2007).

1.3.2.2. BKV and Prostate Cancer

The proliferative nature of the prostate atrophy together with low rate of mutations detected in the tumor suppressor genes Rb1 and p53 of prostate cancer cells (Dong 2006), as compared to other tumors, has suggested for the involvement of an infectious agent that establishes a persistent subclinical infection in the urinary tract, such as polyomavirus BK (Das, Wojno et al. 2008). To sustain this hypothesis there are both the role played by human papillomavirus (HPV) oncoproteins E6 and E7 for the genesis of cervical cancer (Dyson, Howley et al. 1989; Choo and Chong 1993) and the discovery of BKV L-Tag-p53 colocalization to the nuclei of bladder carcinoma cells (Geetha, Tong et al. 2002). On this regard, in an interesting study, Weinreb et al identified a population of patients with polyomavirus infected "decoy" cells in the urine (Weinreb 2006). The data correlate to the incidence of bladder cancer in those "decoy" cells positive patients thus leading to an association between polyomavirus and bladder cancer. In addition, Monini et al. demonstrated the presence of BKV sequences by PCR in more than 50% of both human urinary tract normal and tumor tissues, including prostate (Monini, Rotola et al. 1995). Immunohistochemical analysis of normal and atrophic epithelial prostatic cells using monoclonal antibodies specific to BKV L-Tag has shown that 60% of prostate tissues from healthy subjects are BKV positive as well as 71% of specimens from PIA. In particular, dual staining for L-Tag and p53 has been detected either in the nucleus or in the cytoplasm of PIA specimens but BKV L-Tag and p53 colocalization to the cytoplasm has been particularly seen in prostate cancer cells, while in L-Tag-negative tumors p53 staining was nuclear (Das, Shah et al. 2004). More recently, the same group has demonstrated that the p53 inactivation occurs in atrophic cells expressing wild-type p53 (Das, Wojno et al. 2008), finding promptly supported by other groups (Bocchetta, Eliasz et al. 2008).

Imperiale's group investigation fosters BKV as a cofactor in the etiology of PCa at early stages based on the following "hit-and-run" model: BKV infection of normal epithelial cells drive into PIA. At this level, expression of BKV is evident. The sequestration of wild-type p53 induces infected cells to accumulate enough mutations to growth clonally without control. Both the dilution of BK viral episomes due to lack of replication and the immune selection of BKV infected cell against L-Tag are responsible for the loss of BKV in tumor cells (Das, Wojno et al. 2008). Although a low frequency of BKV L-Tag expression is reported by some authors (Zambrano, Kalantari et al. 2002; Balis, Sourvinos et al. 2007; Bergh, Marklund et al. 2007; Lau, Lacey et al. 2007), their data cannot exclude the possibility for a "hit-and-run" mechanism of BKV oncogenisis. Moreover, because of NCCR hypervariability in tissue cultures (Rubinstein, Schoonakker et al. 1991; Hanssen Rinaldo,

Hansen et al. 2005), genetically rearranged BKV variant might exist in the urinary tract and be responsible for neoplastic transformation in prostate cells (i.e. URO-1) (Shinohara, Matsuda et al. 1993). Thus, given the results obtained with prostate and bladder carcinomas, along with the kidney being the main site for BKV persistence, tumors of the urinary tract are the most logical target sites for an etiological association with BKV.

1.3.3. BKV L-Tag and immune response

During the course of viral infection the host immune response is of central importance in limiting the acute phase as well as in controlling the virus carrier state. The innate immune system is meant to act as a first line defense to prevent viral invasion and replication until development of specific adaptive immune response. Swanson P.A. et al. has recently reviewed the current understanding of the multifaceted immune response invoked by the mouse to face persistent infection in the polyoma virus-mouse model (MPyV) (Swanson, Lukacher et al. 2009). The mouse model of polyomavirus infection has demonstrated that virus control involves both humoral and cellular immune responses. Immunocompetent mice do not clear MPyV, with the virus likely persisting as infectious virus at low levels for the lifetime of the host. Nevertheless, without one or more arms of the adaptive immune response, MPyV infection proceeds unchecked, a consequence being tumor induction. Multiple components of the immune system contribute to limiting MPyV replication, including early induction of T-cell independent antibodies, recruitment of CD4+ and CD8+ T cells, and generation of T-cell dependent humoral immunity. Each of these components has the capacity to reduce viral load on its own, providing several layers of resistance to uncontrolled virus replication. Working together, they assure symptom-free survival with low-level viral persistence.

Serological memory, defined as sustained antiviral antibody levels in the serum following infection, is crucial for protection against re-infection. In persistent infections, serological memory is also essential to check viral replication by decreasing the viral load. Two forms of B cell memory are generated in T-cell dependent humoral responses. B cells that have completed the germinal center reaction become either long-lived terminally differentiated plasma cells that migrate to the bone marrow where they continue to secrete antibodies, or they become memory B cells that reside in secondary lymphoid organs and can rapidly reactivate upon antigen re-stimulation (Ahmed 1992; Ahmed and Gray 1996).
BKV-specific neutralizing antibodies are mostly directed against the major capsid protein VP1, rather than the minor capsid components, VP2 and VP3. Clinical observations suggest

that BKV-specific antibodies may accelerate clearance of primary infection and contribute to protection from BK viremia, but have a minimal role in the containment of polyomavirus-related diseases (Comoli, Binggeli et al. 2006).

The crucial role of T-cell immunity in the control of polyomaviruses infection was first suggested by the indirect evidence of increased incidence of reactivation and clinical disease linked to the degree of immune compromise. Specifically, it was demonstrated that cytotoxic T lymphocytes (CTLs) are critical for the clearance of acute polyomavirus infection in the mouse model, and for the containment of polyomavirus JC-associated progressive multifocal leukoencephalopathy in affected humans (Koralnik, Du Pasquier et al. 2002). Although CD4+ T cells are dispensable for CD8+ T cell expansion and function during the acute phase of the antiviral response, CD4+ T cells are essential for the generation and maintenance of long-lasting, functional memory CD8+ T cells (Sun, Williams et al. 2004; Kemball, Lee et al. 2006). Consistent with this model, there are far fewer MPyV-specific CD8+ T cells in persistently infected CD4+-depleted mice compared to wild type mice (Kemball, Pack et al. 2007).

So far, the study of cellular immune response against BK virus reactivation has been limited to hematopoietic stem cell or solid organ (i.e. kidney) transplantations due to the relevant association between BKV reactivation upon immune suppression and polyomavirus-associated nephropathy (PVAN) inducing allograft failures (Purighalla, Shapiro et al. 1995). Nevertheless, it has become more than ever evident, among immunologists and virologists, the principal role of T cell immune response in balancing BKV reactivation in latently infected cells since it has been seen that BKV-specific humoral immune response alone is inefficient in controlling virus reactivation and spreading and in containing BKV-related diseases (Comoli, Binggeli et al. 2006; Comoli, Hirsch et al. 2008). The finding has been of greater importance when a correlation between reductions of immunosuppressive treatments in PVAN transplanted recipients and decrease of BKV viral load in both plasma and urine along with an increase of frequency of IFN-γ-secreting lymphocytes was proved (Comoli, Azzi et al. 2004). Taking advantage of VP1 and L-Tag overlapping peptide pools and using as readout IFN-γ protein release measured by ELISPOT or flow cytometry assays, several studies have been devoted to the identification of a specific T-cell response to BKV antigens. (Zhu, Rice et al. 1992; Krymskaya, Sharma et al. 2005; Li, Melenhorst et al. 2006; Provenzano, Bracci et al. 2006; Randhawa, Popescu et al. 2006). In particular, Hammer et al. described a relevant CD4+ and CD8+ T cell immune response in transplanted patients beyond a mandatory HLA restriction (Hammer, Brestrich et al. 2006).

Since L-Tag mediated inactivation of p53 has been suggested to represent a critical step for BK viral oncogenicity, (Imperiale 2001; Tognon, Corallini et al. 2003) this antigen

has been identified as an important target for cancer immunity in murine models (Degl'Innocenti, Grioni et al. 2005; Otahal, Hutchinson et al. 2005). Early studies in SV40 infected mice have reported on conserved CD8+ T cells immune responses against SV40 L-Tag (Mylin, Schell et al. 2000). Within the BK virus-cell interaction modalities, L-Tag is thus the most highly expressed BKV antigen in non permissive infected cells and might encompass viral peptides generated by the MHC antigen processing pathway and, possibly, targeted by specific T cells epitopes. In addition, the L-Tag regions required for viral transformation, (pRbs domain aa91-120; p53 domains aa251-470 and aa521-640; almost 80% of the entire sequence) are less likely to be mutated or lost, thus genetically conserved among polyomavirus strains (Velders, Macedo et al. 2001).

A comprehensive understanding of the role of T cell immune response in balancing BKV activity is still demanding. The long-term maintenance of large numbers of specific memory CTL, due to the latency of BKV, with the ability of strongly facing viral infections might result in the clearance of the aggressor. Cellular immune response to BKV is important to define the specific antigenic pattern of the virus. Provenzano et al. have identified two HLA-A*0201-associated BKV L-Tag peptides able to effectively reactivate and expand a T cell memory peptide-specific response in BKV seropositive subjects. In particular, they reported that BKV specific CTL responders to portions of L-Tag exquisitely belong to a CD4+5RA+/CCR7+(-) CD8+ T cell population (Provenzano, Bracci et al. 2006). Notably, the study demonstrated for the first time that BKV seropositive subjects mount a powerful CTL response towards epitopes encompassed by highly phylogenetically L-Tag conserved regions implicated in the p53 mediated control of the cell cycle of host cells.

1.4. T-cell epitope mapping

T-cell epitopes are defined as minimal essential peptide sequences derived from antigenic proteins which are required for recognition of T cells. This recognition is exerted by peptide binding to restricted MHC molecules on professional antigen presenting cells (APC), such as dendritic cells.

Epitopes are thus antigenic portions of a protein and represent the basis for the understanding of adaptive immune response and for the development of preventive vaccines and peptide-based immunotherapies for viral and tumor diseases (Bui, Sidney et al. 2006; Sette and Peters 2007). They can be presented in the context of one or few MHC alleles. The latter supports the identification of promiscuous (i.e. presented by more alleles) (Ho, Mutch et al. 1990; Frahm, Yusim et al. 2007) and/or universal (i.e. presented by most of the alleles) (Panina-Bordignon, Tan et al. 1989; Letourneau, Im et al. 2007) antigenic peptides.

The precise identification of T-cell epitopes has been of importance for the diagnosis and treatment of infectious, allergic, autoimmune and neoplastic diseases (Akdis, Akdis et al. 1996; Stienekemeier, Falk et al. 2001; Lauer, Ouchi et al. 2002; Lazoura and Apostolopoulos 2005; Provenzano, Selleri et al. 2007; Leen, Christin et al. 2008). Identified epitopes within their specific restrictions have been collected to obtain peptide libraries that provide valuable diagnostic tools.

T cell-epitope mapping selects candidate immunogenic HLA-specific peptides within antigenic proteins in order to create large libraries of HLA-restricted immune reagents that are valuable for patient and disease-specific clinical applications. Combinations of these peptides might theoretically fit all potential HLA restricting determinants upon validation of a documented immunogenicity. The past decade has witnessed the search for methodological approaches focusing on the identification of immunogenic peptides and their MHC associations. T-cell epitope mapping can be performed through static methodologies that involve tools, such as MHC-peptide multimers, to define the T-cell precursor frequency to the peptide or dynamic methodologies that analyze functional T-cell response upon peptide induction, such as cytokine gene expression and protein production, T cell proliferation, cytolytic T cell activity and T cell helper function (Kern, LiPira et al. 2005).

Synthetic peptides used for epitope-mapping are generally represented by scrambling protein lysates or peptide pools containing longer sequences encompassing the protein at 8-11 amino-acids pace (Reece, McGregor et al. 1994; Rodda 1996; Rodda 2002). However, investigations using the whole antigen are expensive, laborious and time consuming

procedures. To alternate the above attempts, the “in silico” prediction is the best start for this search and it is now preferred. This computational approach has improved dramatically since it was first developed by Berzofsky, Margalit, and DeLisi in the 1980s (DeLisi 1983; DeLisi and Berzofsky 1985; Margalit, Spouge et al. 1987) and always leads to successful identification of immunogenic peptides. The concurrent use of different prediction algorithms is not only informative on the likelihood of individual peptides to be processed and presented within specific HLA restrictions but it is also able to predict with low margin of error the immunogenic potential of specific sequences. The in silico prediction might also ease the straightforward peptide-pool approach restricting peptide panels by deleting those peptides that have been predicted as poor binders.

The majority of methods for predicting MHC-peptide binding are available for public use (Figure I – 6). They are classified in two groups: methods generated from sequences of peptide binders (sequence-based models), and those that do not require any peptide binding data and are based on the sequence and structure of MHC molecules (modeling-based methods). Sequence-based models can also be divided in two types: those based on qualitative data that describe the ability of peptides to bind to MHC molecules (binders and non-binders) and those trained on quantitative data consisting of peptides whose binding affinity for MHC molecules has been determined. Qualitative models try to recognize the binding pattern that is present on the peptides used for training (binding pattern recognition models), whereas quantitative regression models target the prediction on the actual binding affinities of known peptides to MHC molecules (quantitative binding affinity models).

Despite the prediction of MHC-peptide binding is the basis for anticipating T cell epitopes, it considers all virtual peptides derived from arbitrary cleavage of the protein sequence. The anticipation of peptide fragments that can be actually produced through the intracellular antigen processing can be of remarkable relevance. To that, information from proteolitic activity of the immunoproteasome (protein cleavage into short peptides), peptide transporter (TAP) activity, selection of peptides fitting the groove of MHC molecules and the formation of trimeric complexes (peptide/MHC/β_2-microglobulin) into the lumen of the endoplasmic reticulum (ER) have been modeled and considered for their potential applications for antigen following a HLA class I related endogenous processing (i.e. tumor or viral antigens) (Larsen, Lundegaard et al. 2005) (Brusic, Bajic et al. 2004). Many algorithms have been thus developed combining those information. For instance, one of the best available database integrating proteasomal cleavage, TAP-transport and MHC-binding is the Immune Epitope Data Base (IEDB, http://tools.immuneepitope.org) (Peters, Sidney et al. 2005; Peters and Sette 2007; Vita, Peters et al. 2008; Vita, Zarebski et al. 2010). Nevertheless, MHC-binding prediction, although supported by overall antigen processing

data, does not guarantee in itself that a T-cell bearing such specificity is present indeed in the T-cell repertoire.

MHC class II binding predictions are not as well developed as that of MHC class I binders (Nielsen, Lund et al. 2010). In general, antigenic presentation by HLA class II molecules is employed for peptides following and endocytic processing. Usually, the MHC/peptide complex takes place into cell compartments generated by fusion of early endosomes, transporting the antigenic protein after its phagocytosis/endocytosis, and primary lysosomes operating antigenic degradation and containing the MHC molecule. In contrast to MHC class I, the peptide-binding groove of MHC class II molecules is open (the MHC class II-bound peptides vary widely in length; 9-22 residues), consequently MHC class II molecules impose relatively weak limitations on peptide side chains, harboring broader peptide binding repertoires than MHC class I molecules. These qualities hardly set the generation of reliable peptide binding models for MHC class II molecules (Madden, Gorga et al. 1992; Matsumura, Fremont et al. 1992; Barber and Parham 1993).

In conclusion, T-cell epitope mapping allows identifying immunodominant regions on antigenic proteins. Since methodological approaches currently in use require extended peptide panels and large amounts of T cells, many efforts have been making to develop high throughput techniques, reducing the size of experimental conditions and increasing the sensitivity of the detection of the functional response. In silico prediction of immunogenic peptides combined with *in vitro* and *in vivo* verifications can simplify and accelerate the identification process of T-cell epitopes

1. Binding Pattern Recognition Methods					
Server name	**URL**	**Technique/Algorithm**	**Class**	**nMHC(1)**	**Stypes(2)**
MOTIF_SCAN	http://www.hiv.lanl.gov/content/immunology/motif_scan/motif_scan	Sequence Motifs	I and II	YES	YES
SYFPEITHI	http://www.syfpeithi.de/Scripts/MHCServer.dll/EpitopePrediction.htm	Motif Matrices (MM)	I and II	YES	NO
RANKPEP	http://imed.med.ucm.es/Tools/rankpep.html	Profiles or PSSM	I and II	NO	NO
PEPVAC	http://imed.med.ucm.es/PEPVAC/	Profiles or PSSM	I	YES	YES
EPIMHC	http://imed.med.ucm.es/epimhc/	user made Profiles	I and II	NO	NO
NetMHC	http://www.cbs.dtu.dk/services/NetMHC/	Weight-Matrices	I	YES	NO
ANNPRED	http://www.imtech.res.in/raghava/nhlapred/neural.html	ANN	I	YES	NO
MULTIPRED	http://antigen.i2r.a-star.edu.sg/multipred/	ANN	I and II	YES	YES
MULTIPRED	http://antigen.i2r.a-star.edu.sg/multipred/	pHMM	I and II	YES	YES
SVMHC	http://www-bs.informatik.uni-tuebingen.de/SVMHC/	SVM	I and II	YES	NO
MHC2PRED	http://www.imtech.res.in/raghava/mhc2pred/	SVM	II	YES	YES
POPI	http://iclab.life.nctu.edu.tw/POPI/	SVM	I and II	YES	NO
KISS	http://cbio.ensmp.fr/kiss	SVM	I	YES	NO
2. Quantitative Binding Affinity Methods (regression models)					
Server name	**URL**	**Method/Algorithm**	**Class**	**nMHC(1)**	**Stypes(2)**
BIMAS	http://www-bimas.cit.nih.gov/molbio/hla_bind/	Quantitative Matrices (QM)	I	NO	NO
IEDB	http://tools.immuneepitope.org/analyze/html/mhc_binding.html	ARB-QM	I	NO	NO
IEDB	http://tools.immuneepitope.org/analyze/html/mhc_binding.html	SMM-QM	I	NO	NO
netMHCII	http://www.cbs.dtu.dk/services/NetMHCII/	SMM-QM	II	YES	NO
PROPREDI(3)	http://www.imtech.res.in/raghava/propred1/	QM	I	YES	YES
PROPRED(4)	http://www.imtech.res.in/raghava/propred/	QM (Virtual QM)	II	YES	YES
MHCPRED	http://www.jenner.ac.uk/MHCPred/	QSAR	I and II	YES	NO
netMHC	http://www.cbs.dtu.dk/services/NetMHC/	ANN-regression	I	YES	NO
netMHCpan	http://www.cbs.dtu.dk/services/NetMHCpan/	ANN-regression	I	YES	NO
netMHCIIpan	http://www.cbs.dtu.dk/services/NetMHCIIpan/	ANN-regression	II	YES	NO
IEDB	http://tools.immuneepitope.org/analyze/html/mhc_binding.html	ANN-regression	I	NO	NO
SVRMHC	http://SVRMHC.umn.edu/SVRMHCdb	SVM-regression	I and II	NO	NO
HLABIND	http://atom.research.microsoft.com/hlabinding/hlabinding.aspx	Adaptive Double Threading	I	YES	NO
3. Modeling-Based Methods					
Server name	**URL**	**Method/Algorithm**	**Class**	**nMHC(1)**	**Stypes(2)**
PREDEP	http://margalit.huji.ac.il/Teppred/mhc-bind/index.html	Threading	I	NO	NO
MHC-THREAD	http://www.esd.abdn.ac.uk/~gjlk/MHC-Thread/	Threading	II	NO	NO

(1) The server allows multiple (n) selections of MHC molecules for peptide binding predictions.
(2) The server allows the identification of promiscous MHC-binding peptides and/or prediction of peptides binding to supertypes
(3) PROPREDI QMs were compiled by the authors from the literature.
(4) PROPRED QMs correspond to the TEPITOPE virtual QMs.

Figure I - 6. Freely Available Servers for Public Prediction of MHC-Peptide Binding
(From (Lafuente and Reche 2009)

2. Purpose

The aim of this dissertation was the identification of immunogenic peptides within BKV Large T antigen capable to strongly elicit adaptive memory immune responses to the virus in prostate cancer patients (PCa), irrespective of specific HLA associations, in order to define the level of immunogenicity of these sequences in pre-early and early stages of the disease.

We attempted the characterization of an antigen-specific competent immune surveillance in BKV seropositive PCa patients, gender- and age-matched BPH, gender-matched but not age-matched healthy donors by comparing functional features of peptide expanded CD4+ and CD8+ T cells

The implementation of a L-Tag-specific T cell immune response that favors effective pro-inflammatory activities against BKV in PCa patients might set the stage for original controlled investigations on mechanisms that allow BKV involvement in prostate cancers. It might also provide sound scientific bases for the application of a wide range MHC peptide-vaccination in either preventive or therapeutic settings.

3. Chapters

3.1. MHC-peptide specificity and T-cell epitope mapping: where immunotherapy starts

Maurizio Provenzano, Monica C. Panelli, Simone Mocellin, Laura Bracci, **Giovanni Sais**, David F. Stroncek, Giulio C. Spagnoli and Francesco M. Marincola

TRENDS in molecular medicine 2006 12(10): 465-72

Opinion TRENDS in Molecular Medicine Vol.12 No.10

MHC–peptide specificity and T-cell epitope mapping: where immunotherapy starts

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The evaluation and characterization of epitope-specific human leukocyte antigen (HLA)-restricted memory T-cell reactivity is an important step for the development of preventive vaccines and peptide-based immunotherapies for viral and tumor diseases. The past decade has witnessed the use of HLA-restricted peptides as tools to activate strong immune responses of naïve or memory T cells specifically. This has fuelled an active search for methodological approaches focusing on HLA and peptide associations. Here, we outline new perspective on the emerging opportunity of evaluating HLA and peptide restriction by using novel approaches, such as quantitative real-time PCR, that can identify epitope specificities that are potentially useful in clinical settings.

Epitope mapping: from protein to preventive or therapeutic vaccinations

The discovery of specific epitopes across HLA (human leukocyte antigen) polymorphism is one of the main approaches that investigators take in the development of targeted active antigen-specific immunotherapies. Epitope mapping (see Glossary) is the process of selection of candidate immunodominant HLA-restricted peptides from specific proteins, aiming at the creation of large libraries of HLA-restricted immunogenic reagents that are valuable for patient-specific clinical applications [1]. Combinations of these peptides might theoretically fit all potential HLA-restricting determinants upon validation of a documented immunogenicity.

Several methods have been employed to identify specific HLA-restricted peptides and for the generation of peptide libraries covering the most-common HLA types. By using combinations of these procedures, viral, bacterial and tumor antigens have been studied and several immunogenic peptides have been described. In spite of the large number of immunodominant peptides that have been detected by these techniques, only a minority of them has been used in clinical procedures. For instance, 40 epitopes covering the majority of HLA class I molecules within the main immunogenic cytomegalovirus (CMV) protein pp65 were identified [2]. However, only few are currently used for *in vitro* analysis [3], and especially one – HLA-A2-restricted peptide $pp65_{495–503}$ – is successfully used in clinical settings [4].

The low efficacy of peptides that are used for clinical purposes might be ascribed to: (i) low affinity to the major hystocompatibility complex (MHC) [5]; (ii) low frequency of epitope-specific precursor T cells in individuals bearing defined HLA determinants [6]; or (iii) T-cell anergy that is induced by the lack of professional antigen presenting cells (APCs) [5,7] (Figure 1b). Moreover, proteolysis of peptides by extracellular peptidases might also limit their efficacy [8].

Due to the weak activity of several described peptides, modification of their amino acid sequences, so called epitope enhancement, has been proposed by different groups. Rosenberg *et al.* [9] first enhanced the HLA-A2-restricted peptide that is derived from the gp100 melanoma-associated antigen, $gp100_{209–217}$, for treatment purpose. The synthetic sequence that is called $gp100_{209–2M}$ because of the substitution of Thr2 to Met (ITDQVPFSV versus IMDQVPFSV) increased the affinity to HLA-A2 molecules and generated strong T-cell immune response in almost 90% of the melanoma patients immunized [9]. Similarly, Rivoltini *et al.* [10] modified the HLA-A2-restricted MelanA/$MART1_{27–35}$ peptide (Leu1 to Ala; AAGIGILTV versus LAGIGILTV) that could elicit a robust peptide-specific $CD8^+$ T-cell response due to the increased T-cell receptor (TCR) activation. An amino acid substitution can also inhibit proteolytic activity carried out by serum peptidases [11]. To this purpose, it is convenient to analyse with precision the cleavage-sites that generate peptides within antigens to define the origin of these candidate peptides better [12,13] and, eventually, apply the relevant amino acid substitution [14]. Of course, it is important to demonstrate that T-cell populations that are induced by the synthetic peptide are particularly able to recognize, and eventually lyse, target cells that express the wild-type epitope. Thus, there is an urgent need for the identification of novel antigenic peptides of higher immunogenicity.

The knowledge about the relationship between immunogenicity and HLA binding by peptides is continuously evolving. This evolution needs to be accompanied by the development of technologies that rapidly but reliably

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Available online 7 September 2006.

www.sciencedirect.com 1471-4914/$ – see front matter doi:10.1016/j.molmed.2006.08.008

Glossary

Epitope enhancement: generation of synthetic peptides analogues from wild-type sequences by substitutions of individual amino acid that improve the peptide affinity to MHC molecules at their overall immunogenicity.
Epitope mapping: the process of selection of candidate immunogenic MHC class I and II restricted peptides from characterized proteins.
MHC restriction: recognition of antigenic epitopes by TCR only if they are displayed in the context of defined MHC determinants. It defines T-cell specificity in terms of self MHC molecule that binds peptide fragments within foreign antigens.
Peptide libraries: lists of peptides corresponding to MHC class I and class II restricted T-cell epitopes useful for treatment purposes.

assess T-cell responsiveness with minimal culture times and characterized by high capacity of providing information regarding not only the number of responding lymphocytes but also the quality of the responses that are induced. Techniques currently used for epitope mapping, directly testing MHC–peptide affinities, show major disadvantages such as the inability to detect functional T-cell activities and the limitation to individual HLA alleles [15]. It is thus necessary to use screening tools that directly investigate the ability of HLA-restricted peptides to induce specific immune reactions.

The dogma of MHC–peptide presentation re-stated

T cells can recognize peptides that are embedded into HLA molecules expressed on the surface of APCs. In particular, $CD8^+$ and $CD4^+$ T cells recognize specific peptides when presented in association with HLA class I and class II molecules, respectively [16]. The HLA class I molecules restrict epitopes from endogenously expressed tumor and viral antigens, stimulating cytotoxic immune responses. By contrast, HLA class II antigens restrict cell-mediated immune responses to peptides from exogenous antigens.

Differently from antibody-mediated recognition, the TCR recognizes antigenic epitopes only if presented in the context of MHC–peptide complexes. This is the molecular basis of MHC restriction of cell-mediated immune responses.

Regarding HLA class I restriction, proteins synthesized in the cytosol within cells are degraded to short peptides by the proteolytic activity of the proteasome [17,18]. Selected peptides of ~8–11 amino acids in length are carried into the lumen of the endoplasmic reticulum (ER), assisted by specific carrier proteins such as the transporter associated with antigen processing (TAP) [19]. There, peptides can bind to specific HLA class I heavy chains and β_2-macroglobulin with the help of proteins that operate as intracellular chaperones (calreticulin, ERp57 and tapasin) [20]. This binding gives origin to a trimeric complex that is recognized by TCRs of T cells, particularly when transported on the surface of specialized APCs via the trans-Golgi network [21] (Figure 1a). Recently, *in vitro* studies that have been carried out by using immunoproteasome low molecular protein subunits LMP2, LMP7 and multicatalytic endopeptidase complex subunit MEC-1 [17] have characterized protein sequences that are preferentially recognized [22]. Furthermore, it is also possible to predict peptide affinity to their cognate HLA molecules [23]. Nevertheless, these predictions do not provide direct information on potential peptide immunogenicity [24].

Importantly, the one-to-one correspondence between HLA molecules and peptides has recently been challenged by the discovery that individual peptides can bind to indifferent HLA molecules. This one-to-many correspondence might be due to the cross-binding of different HLA molecules by certain peptides [25–27]. The strict correlation occurring between MHC–peptide binding and the subsequent TCR activation might enable the immediate detection of T-cell responses that are directly based on the cytokine profiles induced. Techniques that rapidly and precisely quantify gene expression using unbiased high-throughput strategies might provide useful tools for large surveys of T-cell epitope immunogenicity in the context of broad HLA variability, by measuring subtle differences in cytokine gene expression that result from peptide-specific stimulation of lymphocytes.

Current methods in use for T-cell epitope mapping

The ability of different peptides to stabilize HLA-A*0201 molecules that are expressed on the human antigen processing defective cell line T2 has been widely assessed by using allele-specific fluorescence-tagged antibodies [28,29]. Candidate peptides identified on the basis of differences in their binding affinities to HLA molecules still need to be evaluated for their specific immunogenicity. In this context, analysis of cytotoxic T lymphocyte (CTL) peptide-specific frequencies by HLA-matched-peptide fluorescent multimers has been considered of relevance for sorting epitope-specific HLA-restricted T-cell populations [30,31], although this method does not provide functional data.

Other investigators tested the ability of specific poly- and oligo-peptides, which were generated by chemical truncation of related proteins, of inducing an immune response [32,33]. These methods usually require repeated *in vitro* stimulations of cells before attempting specific functional assays.

A different technique to identify immune dominant epitopes requires the isolation of mRNA fragments from tumors, the construction of cDNA libraries and their transfection in APCs (usually dendritic cells) bearing the HLA determinants of interest. Cells that are transfected with progressively smaller pools of clones are tested for their ability to induce functional responses in autologous or HLA-compatible lymphocytes. The transfected DNA that elicits the desired response is then identified and sequenced [34]. Dendritic-cell electroporation with viral mRNA [35,36] or gene transfection with viral vectors that encode specific immunogenic sequences [37,38], resulting in epitope recognition by HLA-restricted T cells, have also been successfully used. Although these approaches lead to the definition of candidate immunodominant peptides, they are frequently cumbersome and time consuming both in terms of data acquisition and analysis. Moreover, the peptide ability to induce T-cells immune responses in a HLA-restricted fashion in larger groups of donors is not documented and still needs to be evaluated as commonly addressed by quantifying antigen-stimulated cytokine production.

Thus, methods such as enzyme-linked immunoadsorbent assay (ELISA) [39,40], enzyme-linked

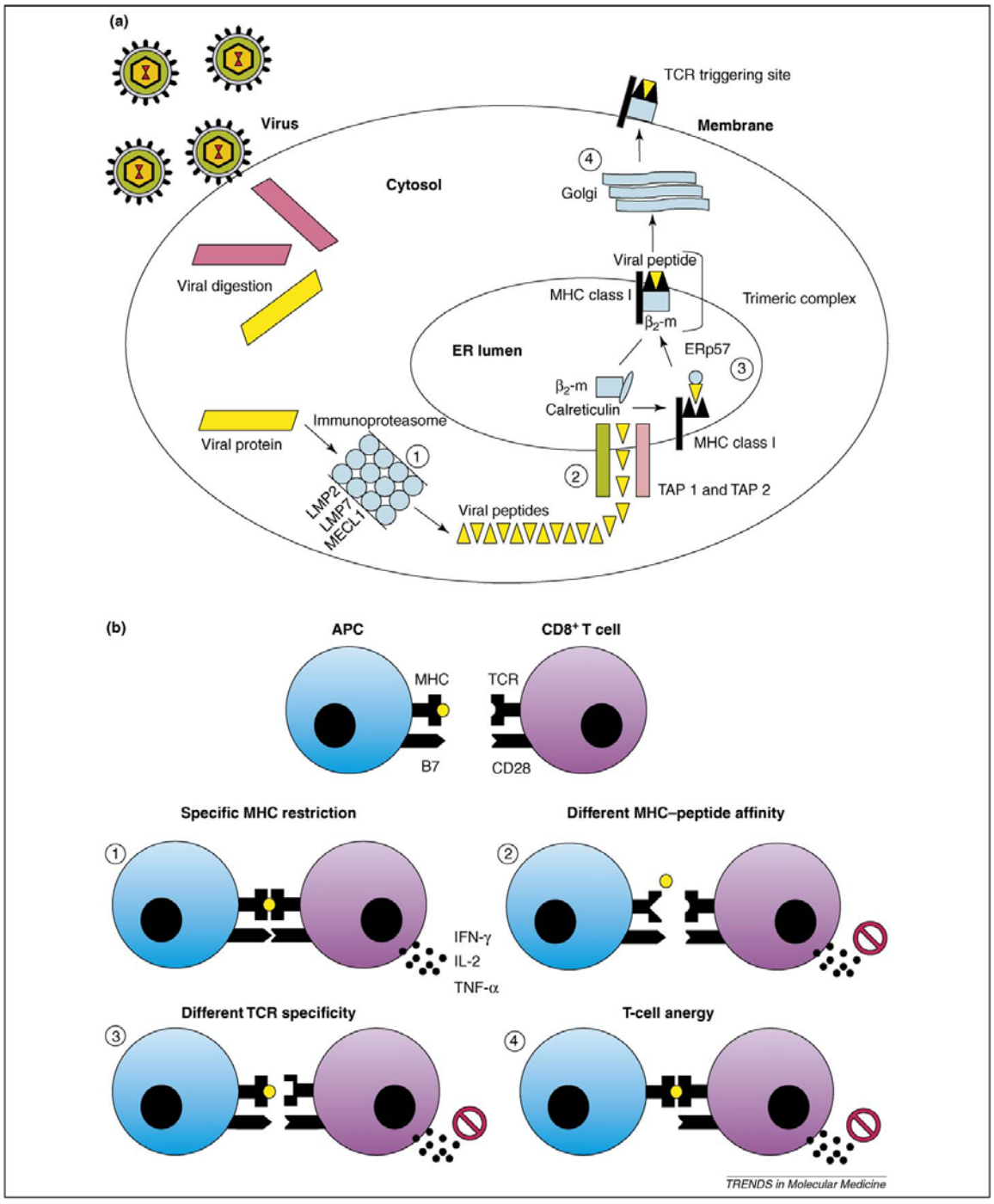

Figure 1. **(a)** The MHC class I antigen processing and presentation pathway. The drawing refers to a viral infection occurring in a professional APC. After the engulfment and digestion of the virions, the derived viral proteins are (1) degraded in the cytosol to short peptides by the proteolytic activity of the immunoproteasome; (2) peptides are carried into the lumen of ER via the peptide transporter TAP; (3) selected peptides fit the groove of MHC class I molecules assisted by various chaperons (i.e. ERp57); and (4) the trimeric complex (class I heavy chain, peptide and β_2-microglobulin (β_2-m)) is transported to the APC surface via the trans-Golgi network. **(b)** TCR triggering by restricted MHC–peptide complexes. The engagement of co-stimulatory molecules is required to enable the induction of cytokine production by the activated T cell. (1) Deficiency of specific MHC restriction can induce to a lack of cytokine production. This might be due to a different MHC–peptide affinity (2) or to a different epitope-specific precursor-T-cell populations (3). No expression of proper co-stimulatory molecules (e.g. B7) leads to T-cell anergy (4).

Table 1. Methods currently in use for epitope mapping

Assay	Advantages	Disadvantages	Clinical relevance
qrt-PCR	Time saving Simultaneous quantification of the expression of different cytokines Indirect measure of T-cell functions Quantitative gene expression	No protein-release quantification No quantitation of specific responding T cells No characterization of T-cell subpopulation Bystander activation	Direct epitope-specific T-cell gene profiling Th1–Th2 bias Suitable for *ex vivo* epitope mapping (T-cell immune response by the definition of different cytokines simultaneously)
ELISA	Protein-release quantification Supernatants can be stored	Bystander activation Low definition for *ex vivo* analysis Single-cytokine assay Requires *in vitro* inductions	Compares analysis of different samples but related to a single cytokine
ELISpot	Protein-release quantification Characterization of T-cell subpopulation	Bystander activation Single-cytokine assay Requires *in vitro* inductions	Functional activity but related to individual MHC–peptide affinities Suitable for *in vitro* epitope mapping (T-cell immune response by the analysis of single cytokine at the time)
MHC-binding	Direct measure of MHC–peptide binding	Requires several steps No TCR-triggering analysis Cumbersome	Not relevant
Multimer staining	Characterization of epitope-specific T-cell subtypes within multimer-sorted cells Possible cytokine analysis	Requires specific multimers Limited to a single epitope Preliminary steps to customize multimers	Epitope-specific T-cell precursor frequency without their functional activity
Flow cytometry	Protein-release quantification Characterization of T-cell subpopulation	Single-cytokine assay Requires several steps Technically sophisticated	Cytokine protein release strictly related to individual MHC restriction Suitable for *ex vivo* epitope mapping (T-cell immune response by the analysis of single cytokine at the time)
mRNA or gene transfection	Innovative Discrimination within MHC alleles	Labor intensive Requires *in vitro* inductions	Direct functional analysis of peptide-induced T cell
Peptide by chemical truncation	Biochemical definition of specific peptides	Labor intensive Requires multiple stimulations	Direct functional analysis of peptide-induced T cell
Cytotoxicity by limiting-dilution assay[a]	Fine identification of CTL precursor frequency	Labor intensive Requires multiple stimulants	Direct functional activity of peptide-elicited T cells

[a]Technique not frequently used for epitope mapping. It is usually employed for functional analysis of antigen-specific T cells after rounds of peptide vaccinations.

immuno-spot technique (ELISpot) [41,42] or intracellular staining (ICS) with cytokine-specific fluorescence monoclonal antibodies [43] are extensively employed in epitope mapping. The initial peptide selection can be entertained using: (i) unbiased libraries of overlapping peptides encompassing full sequences of potentially antigenic proteins under investigation [3,44]; or (ii) powerful biased algorithm programs adopted to rank potential epitopes according to specific characteristics [14,45] and using as read-out specific functional responses of clonal T-cell populations based on characteristic cytokine profiles. These assays, which are quantitative and based on functional responses, usually focus on antigen-stimulated interferon-γ (IFN-γ) production as a reliable, although possibly incomplete, evidence of specific T-cell activation (Table 1).

Quantitative real-time PCR in epitope mapping

The interest in the quantitative evaluation of cytokine gene expression profiles in peripheral blood mononuclear cells (PBMCs) by quantitative real-time PCR (qrt-PCR) to characterize immune responses following *ex-vivo* T-cell stimulation has been increasing in recent years.

This technique was first used to show that in PBMCs that are activated by mitogenic stimuli, such as anti-CD3 antibodies or phytohaemagglutinin (PHA), the expression of genes that encode a large panel of cytokines could be detected [46,47]. Subsequently, it has been used for the immunological monitoring of melanoma patients undergoing antigen-specific active immunotherapy to characterize and quantitate peptide-specific T-cell response [48,49]. Importantly, the simultaneous quantification of the

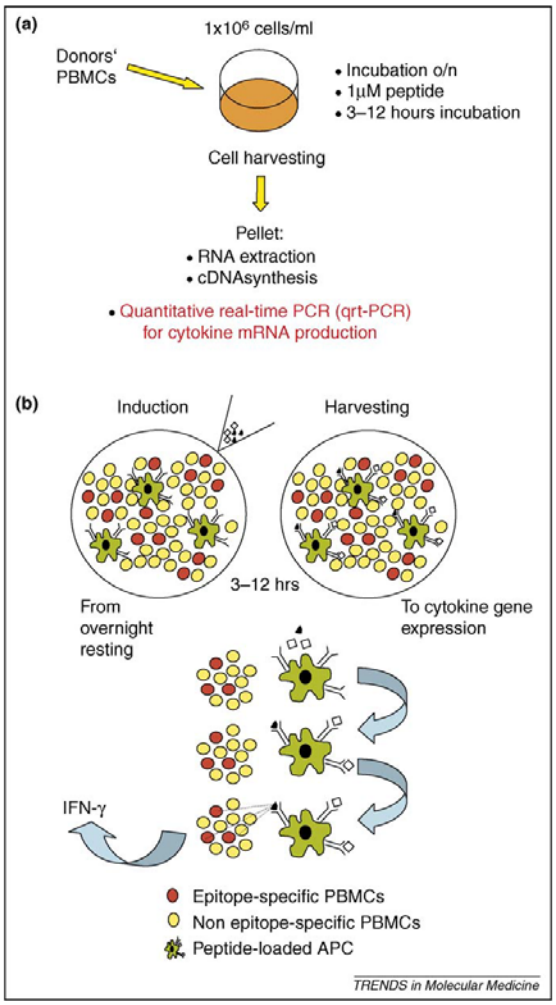

Figure 2. **(a)** PBMCs of viral seropositive and seronegative donors are plated out at a concentration of 1×10^8 cells/ml in a 200 μl medium and incubated overnight (o/n) to reduce IFN-γ expression due to cell manipulation. After starving, cells are either directly stimulated with individual candidate peptides (1 μM) or not stimulated, and qrt-PCR is performed on reverse-transcribed cDNA from total RNA that is isolated at least three hours after stimulation. Three to twelve hours of sensitization are the best interval in which IFN-γ cytokine transcripts can be optimally detected following antigen recall [56]. This wide time range after stimulation is related to variable interactions between some HLA determinants and peptides [74]. **(b)** The main fundament of this method is based on the ability of loaded peptides to recall an immune T-cell activation on HLA-restricted elements within a heterogeneous population. After overnight resting and direct peptide induction, only epitope-specific T cells can recognize the cognate peptide and produce the cytokine.

expression of different cytokine genes including those encoding IFNγ, interleukin-2 (IL-2), IL-4, IL-10, and transforming growth factor-β2 (TGF-β2) before and during vaccination has enabled not only the assessment of specificity but also the characterization of specific functional profiles in T-cell populations from cancer patients [50].

Following extensive evaluation of several technical issues related to the performance of qrt-PCR for different clinical applications [51–54], this method has been also applied for epitope mapping (Figure 2a). Levels of cytokine gene expression in response to *ex vivo* antigen recall were used to detect epitope-specific HLA-restricted T cells in individuals showing evidence of seropositivity towards specific microorganisms [55] (Figure 2b). The main rationale for using *ex vivo* techniques is based on the notion that PBMCs that bear HLA determinants relevant to the HLA–peptide restriction under investigation carry memory T cells that have been exposed *in vivo* to the antigen of interest, either naturally upon viral infection [56] or following specific vaccination procedures [57]. Thus, sensitive assays might be able to identify these responses without *in vitro* cell expansion. The stronger the immune response induced *in vivo* against epitopes from the selected antigen, the easier and more specific should be the documentation of memory T-cell activities.

In this context, methods that are aimed at identifying *ex vivo* immune reactivation, such as ICS and qrt-PCR, are ideally suited to discriminate fine differences that are proportional to the extent of pre-sensitization by accurately quantifying cytokine expression at either protein or molecular level. Ghei *et al.* [58] found that the magnitude of cytokine gene transcription upon peptide stimulation, as analyzed by qrt-PCR in serologically positive donors, represents an accurate index of the biological events following T-cell reactivation that is triggered by specific MHC–peptide complexes. The sensitivity and specificity of this method are decisive in unravelling the fine relationship between MHC–peptide associations and specific immune responses, enabling the characterization of more than one HLA restriction for one individual epitope.

Discrimination among immunogenic HLA–epitope associations

The exquisite sensitivity of qrt-PCR and its ability to quantitate precisely the expression of specific marker genes might provide decisive advantages in the analysis of peptide-specific responsiveness and be particularly informative for the unbiased identification of immunogenic epitopes. Studies on T-cell response that is induced after viral-peptide stimulation have demonstrated differences in T-cell reactivation among HLA-A2-positive donors expressing different HLA-A2 subtypes [59–61]. It has been shown that minor differences between alleles might affect HLA–peptide binding and the subsequent peptide-specific T-cell response [62,63].

The effects of variations among related alleles are readily demonstrated by qrt-PCR. Variability in cytokine mRNA production by T cells from subjects bearing different HLA-A2 subtypes in response to either CMVpp65$_{495–503}$ or influenza virus (Flu) M1$_{58–66}$ peptide stimulation has been demonstrated [64]. Thus, the sensitivity of molecular assessment of T-cell-epitope activity can be employed as a precise read-out because it gives information on the HLA–epitope specificity depending on the exact HLA genotype.

Peptides with multiple HLA associations

The qrt-PCR technology is also useful to clarify unexpected restrictions of specific epitopes rapidly. For example, in a recent study Provenzano *et al.* [65] have reported that stimulation of PBMCs from a HLA-A*2402 0101 subject

with the HLA-A24-restricted CMV peptide $pp65_{341–350}$ induced increased levels of IFN-γ gene expression compared with those induced by specific stimulation of PBMCs from HLA-A*2402 heterozygous subjects that did not carry HLA-A*0101. These results were confirmed by testing PBMCs from many HLA-A*2402 donors expressing or not A*0101, upon exposure to the same peptide. In particular, twofold to threefold increases in IFN-γ gene expression were observed in donors expressing both HLA-A*2402 and A*0101 alleles compared with those in donors lacking the latter determinant. The authors demonstrated that the peptide under investigation can be restricted in its presentation to T cells not only by HLA-A*2402, but also by A*0101 [65]. Further studies carried out with the same technique have documented the ability of $pp65_{341–350}$ to reactivate memory T cells in a broad specificity beyond HLA-A*2401 and A*0101 [58].

In these cases, the degree of peptide affinity to its associated HLA-restriction element is proportionally related to the subsequent peptide-driven T-cell immune activity, as assessed by the magnitude of the production of mRNA-encoding cytokines such as IFN-γ. Interestingly, qrt-PCR can also be used to explore immunodominance in seropositive donors expressing HLA class I alleles that restricts T-cell responses against different peptides or responsiveness to peptides of different sizes.

Advantages and potential pitfalls

The advantage that might make qrt-PCR a more efficient technique over the others described above is that genes encoding several different cytokines can be simultaneously detected. Abdalla *et al.* [66] demonstrated that the kinetics of five cytokines (IL-2, IL-5, IFN-γ, granzyme B and TNF-α) upon stimulation either by mitogen or recall antigen can be defined in the same stimulation run. The final indication is that, regardless of differences among cytokine kinetics, a culture time ranging between 4–24 hours can be successfully used for cytokine profiling. This study in the same stimulation run might provide complete information on both the activation status of armed effector T cells and on T helper (Th)1–Th2 bias.

Limitations in the use of qrt-PCR for epitope mapping should also be considered. The first concern is that gene expression, as revealed by quantitation of mRNA, might not precisely reflect cytokine production as estimated by other techniques (i.e. ICS by flow cytometry or ELISpot). Notably, investigations to validate and confirm the immunogenic capacity of the peptides selected by qrt-PCR are currently in progress. However, cytokine profiles that are established by qrt-PCR are reported to be quantitatively and qualitatively similar to those established by ELISA [67].

A second concern regards the lack of information on the specific cell population primed by selected peptides because, for example, PBMCs include several different cell types that can produce IFN-γ, such as $CD4^+$ or $CD8^+$ T cells, monocytes and natural killer (NK) cells. In this context, it is worth noting that APCs, which are indispensable for antigen-specific T-cell activation, can usually express several cytokines, including IFN-γ. Moreover, a bystander activation of subpopulations that are not directly involved with the HLA–peptide specificity might lead to an overestimation of the final results. To address these issues, marker genes, the expression of which is directly proportional to cell populations of interest (i.e. CD8 or CD4 for T lymphocytes, CD14 for monocytes and CD56 for NK cells) might be used as reference [68]. In contrast, the use of peptides of correct sizes, adequate cell resting time and negative controls are usually sufficient to minimize the unspecific background expression of given genes.

The clinical relevance of T-cell epitope mapping

The clarification of the rules that govern the generation of immunogenic peptides that are active across HLA polymorphisms represents one of the main advances towards the development of epitope-specific vaccination or immunotherapies. The finding that peptides of 8–11 amino acids in length fit in the groove of HLA molecules and, consequently, elicit a restricted T-cell immune activity has prompted their use for specific treatments.

Clinical trials of peptide vaccines or adoptive transfer of peptide-induced T cells have been proposed to elicit T-cell immune responses in combination with adjuvants, including cytokines [69,70], monoclonal antibodies [71] or activation of APC co-stimulatory molecules by a specific T-cell binding [72] (Figure 1b). Peptides are easy to produce and usually stable. Furthermore, they can be used in pools and their immunogenicity can be documented [73].

Molecular oncology and the analysis of the functional roles of distinct viral products in the elicitation of infection and ensuing diseases have helped the characterization of appealing molecular targets for specific immune responses. Retrospective or prospective studies might indicate specific prognostic associations with immune responsiveness to given antigens. Epitope mapping enables the identification of antigenic peptides within the sequences of these target proteins of potential use for vaccination purposes.

HLA-allele-specific algorithms based on MHC-binding affinity studies enable reasonable immunogenicity predictions. However, they might fail to spot: (i) immunogenic peptides with low binding affinities; and (ii) peptides that are characterized by rare HLA restrictions or simultaneously restricted by different alleles.

Box 1. Outstanding questions

- Is the *ex vivo* cytokine gene expression sufficient to inform on the immune activity that is elicited by epitope-specific T cells? Is there a correlation between cytokine gene expression that is induced after an incubation of PBMCs in culture media of few hours and the corresponding epitope-specific cytotoxic activity following *in vitro* cultures?
- Is the *ex vivo* epitope mapping suitable for characterizing specific sequences that might be restricted by several HLA class I and II alleles? Can this method be used for the screening of multiple overlapping and cross-reacting peptides to discriminate among epitopes successfully and to facilitate their identification for particular HLA-restriction elements, independently of the approach originally employed to pre-select them?
- Can qrt-PCR facilitate the identification of immune candidate peptides useful for clinical procedures?

Thus, technologies that allow unbiased epitope mapping, potentially increasing the rate at which immunogenic peptides are identified, would be recommendable. Screening tools that take advantage of pools of peptides encompassing the antigens under investigation and aiming at defining the immediate immune reaction that is elicited by directly stimulating PBMCs, might facilitate the selection of candidate immune peptides that are useful for further clinical investigations.

The clinical relevance of epitope mapping might also be increased by the implementation of technologies that enable the use of small cell numbers, in particular when samples from patients are required.

Concluding remarks

Improvements in *ex vivo* identification of specific memory-T-cell immune reactivation in response to multiple viral antigenic determinants or tumor antigens might lead to a better understanding of the mechanisms that are responsible for anti-viral and anti-tumor immune activity by the rapid identification of novel therapeutic targets (Box 1). Epitope mapping has a key role in this research field.

Its clinical application might require the immediate *ex vivo* detection of T-cell immune responses. In this context, qrt-PCR might have a fundamental role in broadening the list of antigenic determinants beyond those related to frequent HLA molecules that can be used in immunotherapy [74]. The clinical advantage is that a rapid and precise identification of candidate peptides might reduce the time that is needed for peptide identification and enlarge the range of restricting HLA determinants. Furthermore, cytokine gene expression might qualify as high throughput technique, lending itself to automation.

The simplicity with which analysis of large peptide libraries might be performed using an automated approach suggests that in the future large cohorts of individuals bearing informative HLA haplotypes might be tested to increase the understanding of the immunological changes that occur during the development of clinically detectable diseases.

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3.2. A HCMV pp65 polypeptide promotes the expansion of CD4+ and CD8+ T cells across a wide range of HLA specificities

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J. Cell. Mol. Med. 2009 13(8B): 2131-47

J. Cell. Mol. Med. Vol 13, No 8B, 2009 pp. 2131-2147

A HCMV pp65 polypeptide promotes the expansion of CD4$^+$ and CD8$^+$ T cells across a wide range of HLA specificities

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Received: July 10, 2008; Accepted: September 23, 2008

Abstract

Human cytomegalovirus (HCMV) can cause life-threatening disease in infected hosts. Immunization with human leukocyte antigen (HLA)-restricted immunodominant synthetic peptides and adoptive transfer of epitope-specific T cells have been envisaged to generate or boost HCMV-specific cellular immunity, thereby preventing HCMV infection or reactivation. However, induction or expansion of T cells effective against HCMV are limited by the need of utilizing peptides with defined HLA restrictions. We took advantage of a combination of seven predictive algorithms to identify immunogenic peptides of potential use in the prevention or treatment of HCMV infection or reactivation. Here we describe a pp65-derived peptide (pp65$_{340-355}$, RQYDPVAALFFFDIDL: RQY16-mer), characterized by peculiar features. First, RQY-16mer is able to stimulate HCMV pp65 specific responses in both CD4$^+$ and CD8$^+$ T cells, restricted by a wide range of HLA class I and II determinants. Second, RQY-16mer is able to induce an unusually wide range of effector functions in CD4$^+$ T cells, including proliferation, killing of autologous HCMV-infected target cells and cytokine production. Third, and most importantly, the RQY-16mer is able to stimulate CD4$^+$ and CD8$^+$ T-cell responses in pharmacologically immunosuppressed patients. These data suggest that a single reagent might qualify as synthetic immunogen for potentially large populations exposed to HCMV infection or reactivation.

Keywords: peptide cross-presentation • immunotherapy • vaccine

Introduction

Primary infection by human cytomegalovirus (HCMV) is usually mild or asymptomatic and it is effectively controlled by T-cell-mediated immune response in healthy individuals [1]. However, congenital HCMV infection in newborns [2] and viral reactivation in pharmacologically immunosuppressed patients transplanted with solid organs [3] or haematopoietic stem cells (HSCT) [4] are frequently associated with significant morbidity and, eventually, mortality, until the immune system is effectively competent or completely reconstituted [5]. Thus, women in childbearing age and transplant recipients may represent candidate target populations for HCMV active protection.

T-cell-mediated immune response against immunogenic viral targets is of paramount importance due the potential capacity of epitope-specific cytotoxic T lymphocytes (CTLs) to control viral fitness. The administration of major histocompatibility complex (MHC)-restricted immunodominant synthetic peptides to generate CTL immunity preventing the reactivation of HCMV infection represents a promising approach already tested in clinical trials [6]. Interestingly, vaccination of HSCT donors expressing specific HLA determinants might also be important for the generation of HCMV-specific T cells of potential use in adoptive immunotherapy. In this case, healthy virus carriers might represent an important reservoir of antigen-specific CD4$^+$ and CD8$^+$ T cells.

Although much emphasis has been placed on the role of MHC class I restricted CD8$^+$ T cells in the recognition of HCMV-infected cells, there is increasing evidence that CD4$^+$ T cells also have a crucial role in the control of HCMV infection [7].

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doi:10.1111/j.1582-4934.2008.00531.x

The cytotoxic potential of $CD4^+$ T cells specific for viral antigens has been repeatedly documented [8, 9]. Furthermore, transfer of HCMV-specific $CD4^+$ T cells into HSCT has also been found to promote the expansion of HCMV-specific $CD8^+$ CTLs [10] likely due to the helper function exerted by antigen expanded $CD4^+$ T-cell populations [11].

The concomitant use of MHC class I and II restricted peptides would be highly desirable to achieve effective immune responses [12]. However, limitations inherent in MHC diversity and epitope hierarchies within HLA presentation [13] suggest that this approach would require the administration of a panel of immunogenic HLA-binding peptides particularly in case of unknown typing or when rare HLA specificities should restrict immune responses. Ideally, more than one immunogenic reagent should be used [14, 15] for each HLA determinant of interest.

Indeed, a large number of immunodominant HLA class I and II restricted HCMV-derived peptides have been identified [16]. However, only a minority of them has been used in clinical procedures due to the necessity of tailoring immunogens according to specific HLA typing [17].

To address these issues, we attempted the identification of highly immunogenic sequences within HCMV pp65 possibly encompassing both class I and II restricted epitopes using a combination of predictive algorithms. Here we report the functional characterization of a highly immunogenic pp65-derived peptide (RQYDPVAALFFFDIDL; hereafter referred as RQY-16mer peptide) capable of inducing class I and II restricted cytotoxic and lymphoproliferative responses across a wide range of HLA specificities.

Materials and methods

Peptide selection, design and synthesis

Peptides derived from the immunodominant HCMV 65-kD matrix phosphoprotein (pp65; human herpesvirus 5 laboratory strain AD169) were used in this study. Six major algorithms, PAProC (http://www.paproc.de/), MAPPP (http://www.mpiib-berlin.mpg.de/MAPPP/), NetChop (http://www.cbs.dtu.dk/services/NetChop/), Bimas (http://www-bimas.cit.nih.gov/molbio/), SYFPEITHI (http://www.syfpeithi.de) and IEDB (http://www.immuneepitope.org/home.do), were utilized to select candidate immunodominant epitopes within pp65. Furthermore, when indicated, NetMHCIIpan (http://www.cbs.dtu.dk/services/NetMHCpan/) algorithm was also used. Peptides were synthesized by Princeton Biomolecules (Langhorne, PA, USA) with purity ranging from 90% to 100% as analysed by high-performance liquid chromatography, dissolved in 100% Dimethyl Sulfoxide (DMSO) and stored at –70°C until use.

Characterization of immunogenic sequences and algorithm predictions

MAPPP, PAProc and NetChop algorithms were initially used to screen pp65 sequence. Eight peptides, $pp65_{49-70}$, $pp65_{113-127}$, $pp65_{128-144}$, $pp65_{186-208}$, $pp65_{293-305}$, $pp65_{319-330}$, $pp65_{340-355}$ and $pp65_{493-515}$ displayed the highest score of potential immunogenicity, thus extending previous results [18]. These sequences were further analysed for proteasome cleavage sites, TAP transport and MHC binding as evaluated by IEDB, Bimas and SYFPEITHI algorithms. Based on these sets of data, we selected pp65 sequences nesting high numbers of 9mer-peptides restricted by the most frequently represented HLA class I specificities included in the algorithms. $Pp65_{340-355}$ (RQY-16mer) turned out to be the sequence containing the highest numbers of potentially immunogenic HLA class I restricted 9mer-peptides. In particular, six of eight 9mers within RQY-16mer encompassed HLA-A1, A2, A24, A30, A32 and A68 epitope restrictions ($n = 6$) and HLA-B7, B15, B18, B27, B35, B40, B44, B51, B53, B57 and B58 epitope-restrictions ($n = 6$) for a total of 17 valuable HLA class I associations out of the 41 covered by the programs (42%).

Donor selection, cord blood collection, patient accrual, HCMV serology and HLA genotyping

Fourteen HCMV-seropositive and two HCMV-seronegative donors were age, race and sex randomly selected and enrolled in the study upon informed consent (Blutspendezentrum Universitätsspital, Basel, Switzerland). Five umbilical cord blood samples were collected at University Hospital in Basel after either vaginal or caesarean delivery. Ten patients receiving kidney transplant and post-transplantation care were accrued at the Department of Transplantation Immunology and Nephrology, University Hospital in Basel according to a protocol approved by the internal review board (299/06). All patients were treated with an immunosuppressive regimen consisting of mycophenolate, cyclosporine and prednisone, and one underwent prophylactic antiviral treatment, as detailed in 'Results' section. The presence of anti-HCMV antibodies in the serum (anti-pp65 IgG titre) was analysed with passive latex agglutination (CMVSCAN kit, Becton Dickinson Microbiology System, Cockeysville, MD, USA or AxSymTM assay, Abbott, Sligo, Ireland; www.abbott.com). Quantitative real time PCR (qrt-PCR) assays detecting HCMV pp65 DNA in $CD14^+$ cells of healthy donors were performed by using 5′-GTCAGCGTTCGTGTTTCCCA-3′ direct primer, 5′-GGGACACAACACCGTAAAGC-3′ reverse primer and 5′FAM-CCCGCAACCCGCAACCCTTCATG-3′TAMRA fluorogenic probe [19]. Regarding transplanted patients, HCMV-replication was quantified after DNA extraction from ethylenediaminetetraacetic acid (EDTA)-anticoagulated whole blood (Magnapure TM, Hoffman-LaRoche, Basel, Switzerland; www.roche.com) by using 5′-TTTTTTCTAGGCGCTTCCGA-3′ and 5′-ACACTGCGGCTTTGTATTCTTTATC-3′ primers and 5′FAM-AGGCGAAGCCGGCGACGA-3′TAMRA probe at the laboratory of Transplantation Virology, Institute for Medical Microbiology, University of Basel, Switzerland, by taking advantage of previously published amplification protocols [20].

A complete, high resolution, HLA genotyping (HLA-A*, HLA-B* and HLA-DRB1*) was performed by PCR with allele-specific sequencing primers (PCR SSP Protrans, Ketsch am Rhein, Germany) according to the manufacturer's specifications.

T lymphocytes collection, separation and *in vitro* expansion

Peripheral blood mononuclear cells (PBMCs) were separated by Ficoll-Hypaque density gradient centrifugation. T lymphocyte subpopulations ($CD8^+$, $CD4^+$) were purified by magnetic cell separation (Miltenyi Biotech, Bergisch Gladbach, Germany) according to producers' protocols. Cells were then harvested in Roswell Park Memorial Institute (RPMI) medium supplemented with 100 μg/ml Kanamycin, 10 mM Hepes, 1 mM sodium pyruvate,

1 mM Glutamax and non-essential amino acids (all from GIBCO Paisley, Scotland), hereafter referred to as complete medium supplemented with 5% human serum (Blutspendezentrum Universitätsspital, Basel, Switzerland). Both, $CD8^+$ and $CD4^+$ purified T cells were subsequently plated in complete medium with 5% human serum in 24-well plates at a final concentration of 1×10^6 cells/ml and were co-cultured (37°C, 5% CO_2 atmosphere) with irradiated (750 sec. in a gamma ray irradiator equipped with a ^{137}Cs radiation source emitting 100 rad/min.) autologous mature dendritic cells (mDCs) (2×10^5/ml; see below for DCs generation) previously pulsed for 2 hrs with peptides at a final concentration of 10 μg/ml either for priming or for re-stimulation rounds. Recombinant human (rh) interleukin (IL)-2 (Hoffmann-LaRoche, Basel, Switzerland) was added to the cultures at 1 ng/ml, 1 ng/ml and 5 ng/ml, on days 3, 7 and 10, respectively, and cells were restimulated with specific peptides in the presence of irradiated mDCs, as detailed above, on day 7 of culture. In the indicated experiments (see below) control cultures were performed as specified above but in the absence of antigenic peptides.

Dendritic cells generation and CMV infection

For DCs generation, isolated $CD14^+$ (Miltenyi Biotech) were cultured for 5 to 7 days in RPMI complete medium supplemented with 10% foetal calf serum (FCS) (GIBCO Paisley, Scotland), 0.004% β-mercaptoethanol, rhIL-4 (1000 UI/ml) and recombinant human granulocyte macrophage-colony stimulating factor (GM-CSF; 50 ng/ml) to generate immature DCs (iDCs). Maturation of iDCs was induced by exposure to lipopolysaccaride (Abortus Aequi, Sigma-Aldrich, St. Louis, MO, USA) at a concentration of 1 μg/ml.

Immature DCs were infected with cell-free endotheliotrophic (HUVEC) HCMV VR1814 strain at a MOI of 10 and incubated in complete medium supplemented with 10% FCS for 24 hrs in round-bottom 96-well plates at 37°C in a 5% CO_2 atmosphere [21]. As control, iDCs were mock infected with the clarified medium of uninfected HUVEC cultures. Infection grade was measured by both immunofluorescent detection of pp72 and qrt-PCR analysis of pp65 gene expression, as described above (see section 'Donor selection, cord blood collection, patient accrual, HCMV serology and HLA genotyping'). Immunofluorescence analysis was performed on paraformaldehyde-fixed DCs using allophycocyanine-conjugated anti-CD1a (BD Pharmingen, San Diego, CA, USA), or on acetone: methanol (1:1)-fixed iDCs using a goat anti-IE72 monoclonal antibodies (mAb) followed by Alexa Fluor-555-conjugated chicken anti-rabbit antibodies (Molecular Probes, Eugene, OR, USA). Fluorescence was visualized using a 100× Plan Neofluar oil immersion objective (NA 1.3) mounted on a Zeiss Axiovert 100 confocal microscope (Jeha, Germany).

Generation of EBV transformed cell lines

Epstein-Barr virus (EBV)-transformed B-cell lines were generated from each donor by infecting 1×10^6 freshly isolated PBMCs with Epstein-Barr virus containing supernatants from B95.8 cell line. A total of 5 μg/ml cyclosporin A (Novartis, Basel, Switzerland) was added to prevent T-cell response and infected cells were cultured in complete medium with 10% FCS in the presence of rhIL-6 until complete expansion. Cells were then harvested and used for experimental procedures or aliquoted and stored in liquid nitrogen for further use.

Antigenic stimulation and qrt-PCR analysis

Peptide-induced cytokine gene expression was investigated as follows. On day 14 of culture (see above), $CD4^+$ and $CD8^+$ T cells expanded *in vitro* in the presence or absence of antigenic peptide(s) were plated in U-bottom 96-well plates with irradiated autologous mDCs at 10:1 ratio (2×10^5 $CD8^+$ or $CD4^+$ and 2×10^4 mDCs per well). At day 15, after an overnight resting, cells were either peptide-stimulated (1 μg/ml) or left unstimulated. Three hours after cells were harvested for total cellular RNA extraction (RNeasy® Mini Kit Protocol, Qiagen, Basel, Switzerland) and cDNA synthesis (Invitrogen, Carlsbad, CA, USA). Cytokine mRNA transcript amplification was performed as previously described [22] by an ABI prism 7500 FAST sequence detection system using TaqMan® Universal PCR Master Mix Reagents Kit (Applied Biosystems, Rotkreuz, Switzerland) and sets of primers and probes from cytokine genes (interferon [IFN]-γ, tumour necrosis factor [TNF]-α, IL-2, IL-10) already extensively utilized [23]. $CD8^+$, $CD4^+$, $CD14^+$ and β-actin were used as endogenous reference genes [23]. Normalized data were subsequently evaluated as relative quantification. The $2^{-\Delta\Delta Ct}$ method ($\Delta\Delta C_T = [C_T, \text{cytokine} - C_T,\beta\text{-actin}]_{\text{induction}} - [C_T, \text{cytokine} - C_T,\beta\text{-actin}]_{\text{baseline}}$, where C_T is the mean cycle times of the triplicate well readings) was utilized to compute the fold change of cytokine gene expression after peptide induction relative to baseline (unstimulated cells), normalized to endogenous reference genes [24]. When indicated, absolute numbers of cytokine encoding gene copies were calculated as previously described [22].

Cytokine intracellular staining and T-cell phenotype

Peptide expanded $CD4^+$ T lymphocytes (5×10^5) were rested overnight in a 14-ml polypropylene tube (Becton Dickinson, Franklin Lakes, NJ, USA) and then stimulated with peptide-pulsed (10 μg/ml) mDCs at a final concentration of 5×10^4 per each tube. One hour after cell activation, 10 μg/ml of Brefeldin A (Sigma, Saint Louis, MI, USA) was added. After a 5-hr additional incubation, cells were transferred to 5-ml round-bottom tubes (Becton Dickinson) and cell incubation was stopped by washing cells in 2 ml cold phosphate-buffered saline (PBS) for 5 min. Pellets were re-suspended in 1 ml PBS containing 1 mM EDTA and 0.5% FCS and cells were extracellularly stained with 10 μl of fluorescent mAb recognizing CD3, CD4 and CD8 (BD Bioscience, San Jose, CA, USA) for 15 min. at 4°C in the dark. Cell fixation was performed with 2 ml of BD FACS Lysis Solution (BD Bioscience) according to the producers' instructions. Cellular permeabilization was performed by re-suspending cells in 500 μl of FACS Permeabilizing Solution 2 (BD Bioscience) at a 1:10 dilution in Diethyl Pyrocarbonate (DEPC) water at room temperature for 10 min. For intracellular staining, cells were stained with either 10 μl of human anti-IFN-γ-FITC, TNF-α-Fluorescein Isothiocyanate (FITC), IL-2-FITC mAbs or mouse IgG_1 isotype-FITC (BD Bioscience) and incubated for 30 min. at 4°C in the dark. Samples were analysed on a FACSCalibur flow-cytometer equipped with Cellquest software (Becton Dickinson, San Jose, CA, USA).

^{3}H-Thymidine incorporation assays

Isolated $CD4^+$ T cells from freshly separated PBMCs were cultured in complete medium with 5% human serum in flat-bottom 96-well plates (Becton Dickinson, Le Pont de Claix, France) at 2×10^5 cells/well and stimulated with peptide-pulsed mDCs. On day 5, ^{3}H-thymidine (Amersham, Little Chalfont, UK) was added at 1 μCi per well. After an 18-hr incubation, cells were harvested and tracer incorporation was measured by beta counting. Data were expressed as ^{3}H-thymidine incorporation in counts per minutes (cpm).

Caspase-3 production by dendritic cell

Caspase-3 release was carried out by using PhiPhiLux® (Oncoimmunin Inc, Gaithersburg, MD, USA) as follows. Infected iDCs were suspended in complete medium with 10% FCS at 1×10^6/ml. Cells were then incubated at 37°C, for 1 hr in the presence of 3 M specific dye from CyToxiLux Oncolmmunin (CTO™; Molecular Probes) and peptides (1 μg). The cells were then washed once and re-suspended in complete medium with 10% FCS at 1×10^6/ml. For each condition, 2×10^6 of 2-week peptide stimulated $CD4^+$ effector-cells were co-cultured with 2×10^5 CTO stained target cells (infected or control iDCs) in round-bottom 96-well plates (Becton Dickinson) for 2 hrs at 37°C. The supernatant was then removed and the cells were incubated in 75 μl/well of the indicated caspase-3 substrate (10 M, Oncolmmunin, Gaithersburg) for 30 min. at 37°C and washed twice with PBS [25]. An extra staining for CD1a was performed to identify iDCs. Cells were then analysed by flow cytometry (FACSCalibur; Becton Dickinson) using Cell Quest software (Becton Dickinson) and gated for CD1a expression.

Cytotoxicity assays

Cytotoxic activity of peptide expanded $CD8^+$ T cells was tested on day 14 by 4 hrs chromium release assays using, as targets, autologous EBV-transformed B cells previously labelled with ^{51}Cr (50 μCi of ^{51}Cr for 1 hr at 37°C) and pulsed for 2 hrs with cognate or control peptides at a concentration of 2.5 μg/ml. Specific lysis of target cells from triplicate wells was calculated according to the standard formula: 100 × ([cpm experimental release – cpm spontaneous release]/[cpm maximal release – cpm spontaneous release]). Results are reported as mean of delta specific lysis (ΔSL; experimental positive release – experimental negative release) [26].

T lymphocytes multimer staining

HLA-A*0201 and HLA-A*2402 MCH Pro5™ PE-labelled pentamers for $pp65_{340-348}$ (RQY-9mer) and $pp65_{341-349}$ (QYD-9mer) (Proimmune, Oxford, UK) were used for surface staining of cells under investigation. Cells were first stained with 1 μl MHC PE-labelled pentamer for 15 min. at 4°C in the dark. Subsequently, they were washed once and stained with 5 μl of CD8-FITC (BD Bioscience) for 30 min. at 4°C in the dark. After staining, samples were analysed on a FACSCalibur flow-cytometer equipped with Cellquest software (Becton Dickinson).

Glutaraldehyde fixation

Immature DCs were fixed as described by Shimonkevitz *et al.* [27]. Briefly, cells were re-suspended in PBS and fixed by addition of glutaraldehyde (final concentration 0.05%) for 30 sec. at room temperature. The reaction was stopped by adding L-glycine (final concentration 0.2 M) for 45 sec. and by three washes with PBS.

For the measurement of antigen-specific T-cell activation, 5×10^4 fixed or unfixed autologous iDCs were co-cultured in U-bottom 96-well plates with 2×10^5 antigen specific *in vitro* expanded T cells for 3 hrs in the presence of the indicated peptides. T-cell reactivation was evaluated by IFN-γ gene expression.

Statistical analysis

Statistical analysis was performed with Prism 4 (GraphPad) software. Data were reported as mean ± S.D. or as median and ranges where appropriate. Categorical markers were analysed by Pearson's chi-square test. A two-tailed paired t-test was used to calculate *P*-values. Due to variability related to heterogeneous HLA restriction, unpaired t-tests and F tests were used to compare variance when appropriate. Differences were considered significant at $P < 0.05$, CI 95%.

Results

Both $CD4^+$ and $CD8^+$ T cell respond to RQY-16mer peptide

In order to define the overall immunogenicity of RQY-16mer, we evaluated its capability to elicit $CD4^+$ and/or $CD8^+$ immune responses in T cells. $CD4^+$ and $CD8^+$ T cells from either HCMV-seropositive or seronegative donors (Table 1A) were 2-week *in vitro* stimulated using RQY-16mer peptide-loaded or untreated autologous mDCs. A 3-hr assay of RQY-16mer mediated induction of IFN-γ, TNF-α, IL-2 and IL-10 cytokine gene expression was then performed on expanded cells. A cut-off level of responsiveness (= 1.9 folds) was calculated based on the responses to 3 hrs stimulation of 2-week cultures from seropositive donors conducted in the absence of antigenic peptide.

RQY-16mer induced the activation of T cells pre-cultured in the presence of the peptide from all HCMV-seropositive donors, as detectable by a significant increase in pro-inflammatory cytokine gene expression as compared to unstimulated controls, irrespective of their HLA specificities (Fig. 1A). In particular, IFN-γ gene expression was increased 6.27 ± 2.96 folds in RQY-16mer stimulated $CD4^+$ T cells, as compared to control cultures. TNF-α and IL-2 gene expression were also induced accordingly (6.83 ± 4.67 folds and 8.141 ± 0.85 folds, respectively). In contrast, no concomitant induction of IL-10 gene expression was detectable (0.83 ± 0.44 folds; IFN-γ *versus* IL-10 gene expression $P = 0.001$; TNF-α *versus* IL-10 gene expression $P = 0.02$; IL-2 *versus* IL-10 gene expression $P = 0.0001$).

Most intriguingly, expression of IFN-γ, TNF-α and IL-2 genes was also induced by RQY-16mer stimulation in purified $CD8^+$ T cells putatively responding only to shorter HLA class I restricted peptides. Indeed, the expression of these genes was increased 5.11 ± 2.94 folds, 6.99 ± 2.45 folds and 5.62 ± 0.95 folds, respectively in RQY-16mer stimulated $CD8^+$ T cells from HCMV-seropositive donors, in comparison to unstimulated controls. Again, no induction of IL-10 gene expression was observed (1.28 ± 0.61 folds; IFN-γ *versus* IL-10 gene expression $P = 0.02$; TNF-α *versus* IL-10 gene expression $P = 0.004$; IL-2 *versus* IL-10 gene expression $P = 0.009$, Fig. 1A).

Notably, cytokine gene expression in HCMV-seronegative donors was not significantly increased following RQY-16mer

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Table 1 HLA typing and HCMV serology of donors and cord blood specimens

A	Donor	HLA-A*	HLA-B*	HLA-DRB1*	HCMV serological testing (i)	HCMV molecular molecular testing (ii)
	D1	0201,0201	4901,5101	14,16	pos	pos
	D2	0101,0201	0801,5101	03,15	pos	pos
	D3	0201,0301	15,4001	1302,1302	pos	pos
	D4	0101,0201	0501,4402	07,11	pos	pos
	D5	0201,0301	2701,4402	04,16	pos	pos
	D6	1101,2402	0702,5701	07,15	pos	pos
	D7	0212,0301	0709,1801	1301,15	pos	pos
	D8	0101,0301	1401,5701	04,07	pos	pos
	D9	2402,3001	0702,0801	03,15	pos	pos
	D10	0201,0201	0702,1501	04,12	pos	pos
	D11	1101,2402	35,53	0101,1302	pos	pos
	D12	0201,3201	0801,5801	03,03	pos	pos
	D13	02 ,6801	5101,55	04,14	pos	pos
	D14	0201,0201	35,55	11,14	pos	pos
	D15	0201,2301	5101,5301	07,14	neg	neg
	D16	0201,0301	1302,1501	1301,15	neg	neg
B	**Cord blood**	**HLA-A***	**HLA-B***	**HLA-DRB1***	**HCMV serological testing (i)**	**HCMV molecular testing (ii)**
	CB1	0101,0201	0702,4001	13,15	na	na
	CB2	2402,1101	15,4001	07,11	na	na
	CB3	0201,0301	1506,1801	14,16	na	na
	CB4	0201,0201	4001,4601	07,14	na	na
	CB5	0101,0201	0702,5101	01,03	na	na

(i) Specific anti-pp65 IgG titre. Pos = agglutination >1:8 dilution
(ii) qrt-PCR amplification of pp65 DNA in $CD14^+$ cells.
na = not available

stimulation of $CD4^+$ or $CD8^+$ T cells, in comparison with unstimulated cells, and its variations did not reach the calculated cut-off level of 1.9 folds.

Importantly, a highly significant correlation between the extent of the expression of IFN-γ and TNF-α genes in $CD4^+$ or $CD8^+$ T cells upon RQY-16mer peptide induction and the amount of pp65 DNA transcripts in $CD14^+$ monocytes from the same donors was also observed ($CD4^+$/IFN-γ: $r = 0.797$, $P = 0.0004$; $CD4^+$/TNF-α: $r = 0.710$, $P = 0.003$; $CD8^+$/IFN-γ: $r = 0.734$, $P = 0.002$; $CD8^+$/TNF-α: $r = 0.696$, $P = 0.004$; Fig. 1B–E).

To further support the notion of an antigen-specific responsiveness to RQY-16mer, unrelated to unspecific mitogenic effects, five cord blood specimens expressing a variety of HLA class I and II molecules (Table 1B) were collected for *ex vivo* IFN-γ gene expression upon peptide stimulation. Four specimens were from HLA-A*0201 donors. RQY-16mer failed to induce significant increases in IFN-γ gene expression in these samples. In contrast, positive control phytoemagglutinin (PHA) induced a strong cytokine gene expression ($P = 0.0001$; Fig. 1F). As expectable in cord blood T cells, control virus derived HLA-A*0201 restricted peptides selected among latent (CMV $pp65_{495-503}$ [28], BKV $LTag_{579-587}$ [26], EBV $LMP1_{159-167}$ [12]) and non-latent viruses (Flu $M1_{58-66}$ [29]) were also unable to induce cytokine gene expression.

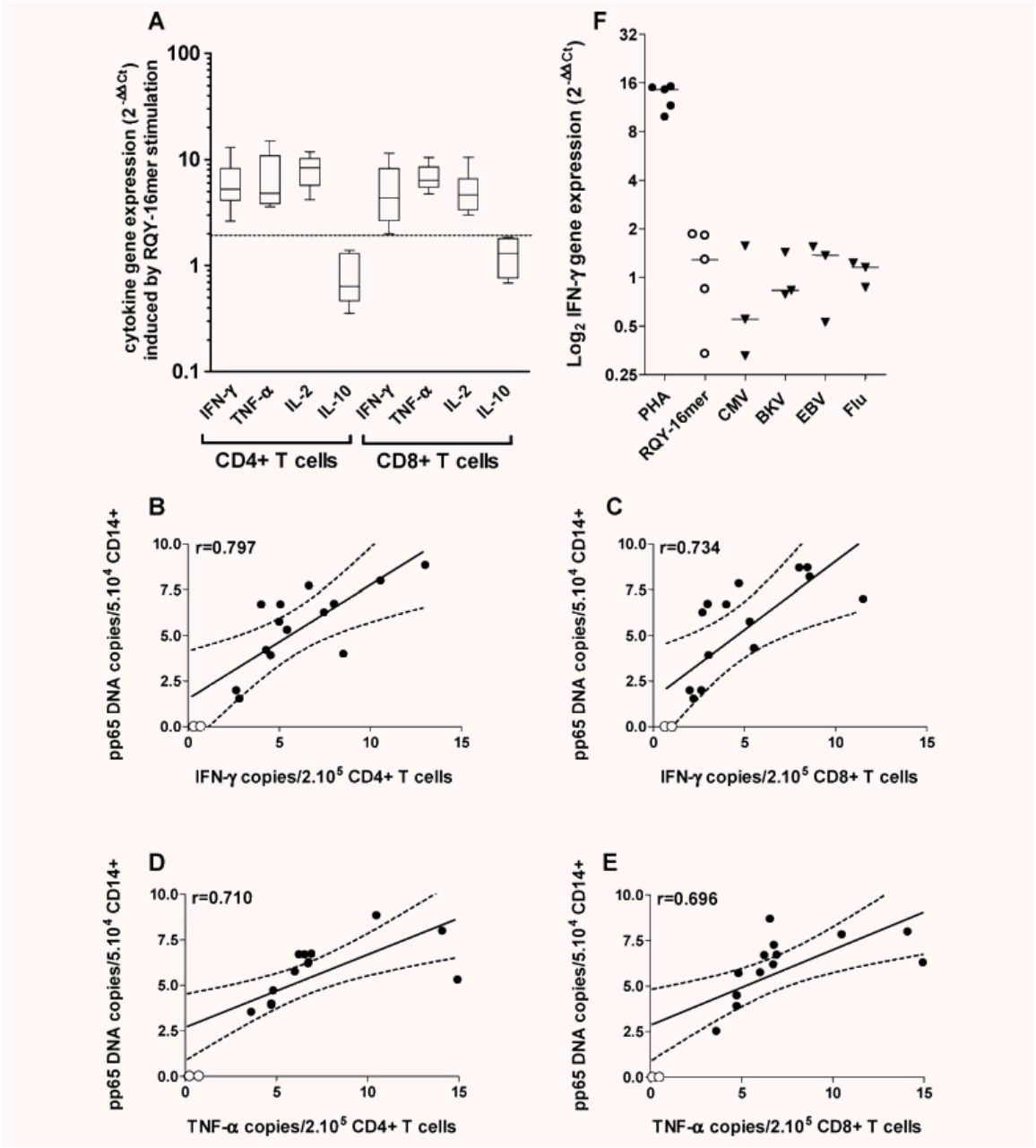

Fig. 1 Responsiveness of $CD4^+$ and $CD8^+$ T cells to immunostimulation by RQY-16mer peptide irrespective of HLA typing. (**A**) $CD4^+$ and $CD8^+$ T cells from 14 seropositive healthy donors were cultured for 2 weeks in the presence of RQY-16mer peptide, as detailed in the 'Materials and methods' section. Peptide-induced expression of the indicated cytokine genes was then assessed following a 3-hr incubation in the presence or absence of RQY-16mer. Data are reported as cytokine gene relative quantification ($2^{-\Delta\Delta Ct}$) in RQY-16mer triggered cells, as compared to cells incubated in the absence of peptide. Interquartile ± S.D. are reported for each single cytokine gene expressed by either $CD4^+$ or $CD8^+$ T cells upon RQY-16mer induction. Negative cut-off (dotted line) was set at 1.9-fold increase based on average variation of cytokine gene expression in T cells cultured in the absence of

antigenic peptide for 2 weeks and then challenged for 3 hrs in the presence or absence of RQY-16mer peptide. RQY-16mer stimulation of T cells from seronegative donors ($n = 2$) failed to induce increases in cytokine gene expression exceeding cut-off values (see panels **B–E**, empty circles). (**B–E**) Specific RQY-16mer T-cell reactivation as an hallmark of HCMV latency. Numbers of IFN-γ and TNF-α gene transcript copies induced by RQY-16mer stimulation in either $CD4^+$ or $CD8^+$ cultured T cells were correlated to those of pp65 DNA copies detectable in autologous fresh $CD14^+$ cells for each of the 14 HCMV-seropositive (full circles) and two HCMV-seronegative (empty circles) donors under investigation. Categorical markers were analysed by Pearson's chi-square test. A two-tailed paired t-test was used to define *P*-values (**B**) $CD4^+$/IFN-γ: $P = 0.0004$; (**C**) $CD8^+$/IFN-γ: $P = 0.002$; (**D**) $CD4^+$/TNF-α: $P = 0.003$; (**E**) $CD8^+$/TNF-α: $P = 0.004$). (**F**) Antigen-specific responsiveness to RQY-16mer is not related to unspecific stimulatory effects. IFN-γ gene expression was studied in cells from five cord blood specimens upon *ex vivo* RQY-16mer peptide stimulation (median 1.28 folds, range 0.33–1.85) or, in HLA-A*0201 cord blood specimens ($n = 3$) following stimulation with different HLA-A*0201 restricted virus derived antigens (HCMV $pp65_{495-503}$, BKV large T antigen $_{579-587}$, EBV LMP-$2_{426-434}$, influenza matrix$_{58-66}$; median 1.01, range 0.32–1.57) as compared to mitogenic phytoemagglutinin stimulation (median 14.52 folds, range 9.85–15.14; $P = 0.0001$). Data are reported as IFN-γ gene expression relative quantification ($2^{-\Delta\Delta Ct}$) as compared to cells from the same donors pre-incubated in the absence of stimuli.

$CD8^+$ T-cell reactivity to nonamer (9mer)-peptides nested within RQY-16mer peptide

Cytokine gene expression data indicated that RQY-16mer peptide was able to efficiently stimulate $CD8^+$ T cells, in spite of its relatively large size. We explored in more detail the ability of each of the eight 9mer-peptides tiled at one amino acid pace within RQY-16mer peptide to recall epitope-specific immune responses from $CD8^+$ T cells previously expanded upon RQY-16mer induction.

Representative results from three donors (D9, D10 and D11), expressing HLA class I alleles accounting for >60% of the alleles expressed in our study cohort and detectable with high frequency in different populations are reported (Fig. 2A). At least four 9mer-peptides were found to be able to stimulate significant IFN-γ gene expression in cells from these donors. Interestingly, the 9mer peptides inducing IFN-γ gene expression in cells from specific donors were frequently predictable based on the IEDB algorithm. For instance, according to the IEDB MHC binding score (IC_{50}), peptide RQY-9mer could be restricted by several alleles including, *i.e.* HLA-A*0201, A*3001, A*3201 HLA-B*0702, B*1501, B*2701, B*4001. Indeed, among the donors tested in the experiments, as reported in Fig. 2, this peptide induced significant IFN-γ gene expression in cells from the two donors (D9 and D10) bearing one or more of these specificities, but its immunogenic activity was barely detectable in the donor (D11) lacking the expression of these alleles (Fig. 2A).

Table 2 cumulatively reports predicted HLA restricting determinants and specific scores of 9mer peptides encompassed by RQY-16mer. Numbers of donors expressing defined HLA alleles and showing evidence of specific response are also displayed. Notably, considering the redundancy of the restriction of the presentation of some of the peptides under investigation, we are unable to unequivocally attribute responsiveness to restriction by individual allelic products. Nevertheless, it is of interest that at least six of eight peptides nested within the RQY-16mer sequence were able to induce specific responses. Furthermore, two peptides (RQY- and ALF-9mers) fulfilled immunodominance criteria, as defined by Nastke *et al.* [30], in that they were able to stimulate specific responses in at least six of eight donors (75%) expressing HLA-A*0201. The previously described immunodominant QYD-9mer [31] was also found to be able to stimulate specific responses in three of three HLA-A*2401 donors, thereby confirming the integrity of our experimental approach.

These findings were supported by cytotoxicity assays performed by using RQY-16mer peptide expanded CTLs from the three representative donors under investigation as effectors and autologous EBV-transformed B cell loaded with specific HLA class I restricted matching peptides as targets. Significant levels of specific lysis at 10:1 E/T ratio ($\Delta SL_{10:1}$) could be observed by pulsing target cells with 9mer-peptides capable of inducing IFN-γ gene expression (Fig. 2B).

Comparative analysis of $CD8^+$ T-cell re-activation by RQY-16mer and RQY- or QYD-9mers induction

To further characterize the $CD8^+$ T-cell stimulating capacity of RQY-16mer, multimeric reagents inclusive of HLA-A*0201 or HLA-A*2402 and immunodominant RQY- or QYD-9mers, respectively, were generated. These tools allowed to *ex vivo* stain sizeable percentages of $CD8^+$ T cells from HCMV-seropositive donors expressing specific HLA alleles (Fig. 2C and F). T cells from the two donors (D9 and D10) were co-cultured with autologous mDCs in the presence of RQY-16mer or RQY- or QYD-9mers peptides. Multimer stainings and specific cytotoxic activities were then evaluated. Predictably, RQY- or QYD-9mers were highly effective in expanding specific $CD8^+$ T cells endowed with cytotoxic capacity (Fig. 2D and G). Most interestingly, RQY-16mer was also effective in expanding $CD8^+$ T cells with similar phenotypic and functional features (Fig. 2E and H).

Stimulation of both $CD4^+$ and $CD8^+$ T-cell responses by RQY-16mer requires active processing by peptide-pulsed DCs

We hypothesized that RQY-16mer could be fully internalized into DCs, processed through an exogenous pathway and cross

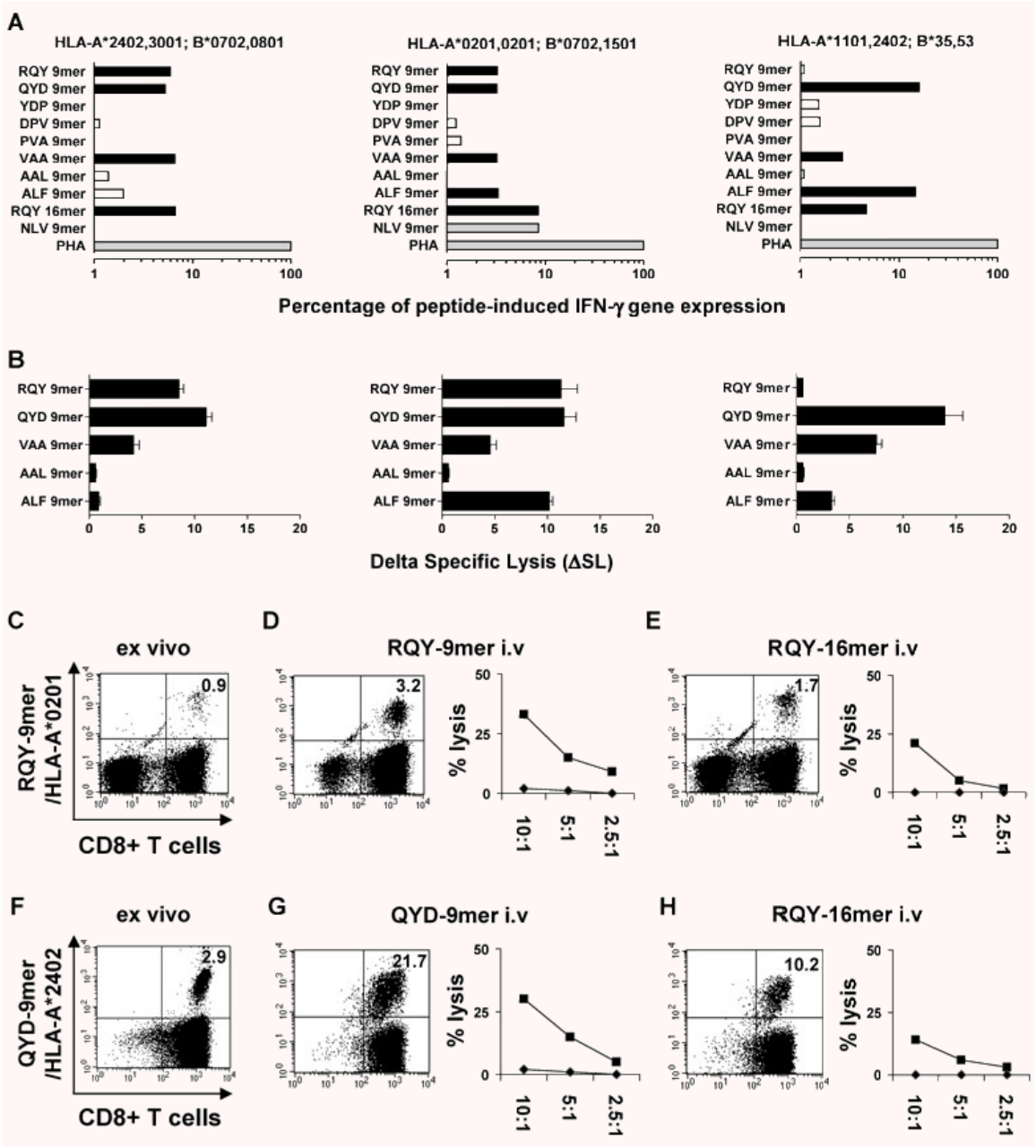

Fig. 2 Peptide-specific immune stimulation induced by 9mer-peptides nested within RQY-16mer in $CD8^+$ T cells following *in vitro* expansion driven by RQY-16mer. (**A**) $CD8^+$ T cells from representative HCMV seropositive healthy donors (D9-D11, see Table 1) expressing the indicated HLA class I specificities were cultured in the presence of RQY-16mer, as detailed in the 'Materials and methods' section. Cells were then stimulated for 3 hrs in the presence of 9mer-peptides encompassed within RQY-16mer sequence, and cytokine gene expression was assessed by qrt-PCR. Data are reported as percentage of IFN-γ gene expression, as compared to positive control phytoemagglutinin (PHA). Black bars identify stimulatory 9mer-peptides inducing cytokine gene expression >2% of PHA triggered levels. The HLA-A*0201 restricted $pp65_{495-503}$ (NLV) peptide was used as control. (**B**) Cytotoxic

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activity of RQY-16mer stimulated $CD8^+$ T cells against nested 9mer-peptides. $CD8^+$ T cells from the same experiments depicted in A were tested as effector cells in ^{51}Cr release assays by using, as targets, autologous EBV-transformed B cells upon pulsing with the indicated peptides. Data are reported as mean of delta specific lysis (ΔSL) $\pm$ S.D. at 10:1 E/T ratio. (**C–H**) Frequencies of HCMV epitope specific cells *ex vivo* or following peptide-driven *in vitro* (i.v.) T-cell expansion. Total T cells (**C–E**) or purified $CD8^+$ T cells (**F–H**) from two HCMV-seropositive donors (D10 and D9, respectively) expressing appropriate HLA specificities were stained *ex vivo* with RQY-9mer/HLA-A*0201- (**C**) and QYD-9mer/HLA-A*2402-pentamers (**F**). Cells were considered positive if their mean fluorescence intensity (MFI) exceeded by at least 10-fold that of multimer negative $CD8^+$ cells. The same cell preparations were then stimulated for 2 weeks in the presence of RQY-9mer (**D**), QYD-9mer (**G**), or RQY-16mer (**E**, **H**) and staining with RQY-9mer/HLA-A*0201- and QYD-9mer/HLA-A*2402-pentamers was repeated on cultured cells. Cells expanded in the presence of specific 9mer-peptides or RQY-16mer were also used as effector cells in cytotoxicity assays utilizing, as targets autologous EBV-transformed B cells upon pulsing with specific (squares) or a control ($gp100_{280-288}$, HLA-A*0201 restricted melanoma associated epitope, diamonds) peptide. Data are reported as percentage-specific lysis at the indicated E/T ratios. Standard deviations, never exceeding 10% of the reported values, were omitted.

Table 2 Algorithm predictions and responsiveness to 9mer-peptides nested within RQY-16mer

HLA class I alleles (i)	9 mer-peptides restricted by HLA specificities listed in column 1 (ii)	IEDB score (iii)	MHC affinity IC50[nM] (iv)	number of responding donors out of those bearing the allele (v)
HLA-A*0101 ($n = 3$)	QYDPVAALF	−1.26	4812.7	2/3
HLA-A*0201 ($n = 8$)	RQYDPVAAL	−0.36	264.6	6/8 (75%)
	VAALFFFDI	−1.74	683.1	5/8
	ALFFFDIDL	−0.13	99.4	6/8 (75%)
HLA-A*2402 ($n = 3$)	QYDPVAALF	0.17	178.7	3/3
	YDPVAALFF	−0.73	1706.5	1/3
	DPVAALFFF	−0.14	214.6	1/3
	VAALFFFDI	−1.84	858.2	1/3
HLA-A*3001 ($n = 1$)	RQYDPVAAL	−1.09	1426.9	1/1
HLA-A*3201 ($n = 1$)	RQYDPVAAL	0.6	29.3	1/1
	QYDPVAALF	-0.33	572.9	0/1
	ALFFFDIDL	1.04	6.7	1/1
HLA-A*6801 ($n = 1$)	DPVAALFFF	−0.26	285.9	1/1
HLA-B*0702 ($n = 3$)	RQYDPVAAL	−1.15	1640.8	1/3
HLA-B*1501 ($n = 2$)	RQYDPVAAL	0.12	88.9	2/2
	YDPVAALFF	−0.98	3044.4	1/2
HLA-B*1801 ($n = 1$)	YDPVAALFF	−0.78	1920	0/1
	DPVAALFFF	0.15	110.1	0/1
HLA-B*2701 ($n = 1$)	RQYDPVAAL	−0.91	941.7	0/1
HLA-B*3501 ($n = 2$)	YDPVAALFF	−0.62	1312.1	0/2
	DPVAALFFF	0.45	55.4	0/2
	ALFFFDIDL	−3.44	3926.3	1/2
HLA-B*4001 ($n = 1$)	RQYDPVAAL	−0.74	635.6	0/1
HLA-B*4402 ($n = 2$)	DPVAALFFF	−1.38	3733.4	1/2
HLA-B*5101 ($n = 3$)	DPVAALFFF	−0.99	1513	1/3
	VAALFFFDI	−1.65	558.9	1/3

Table 2 Continued

HLA class I alleles (i)	9 mer-peptides restricted by HLA specificities listed in column 1 (ii)	IEDB score (iii)	MHC affinity IC50[nM] (iv)	number of responding donors out of those bearing the allele (v)
HLA-B*5301 ($n = 1$)	DPVAALFFF	−0.2	246.6	0/1
	VAALFFFDI	−1.8	794.1	1/1
HLA-B*5701 ($n = 2$)	VAALFFFDI	−2.05	1407.9	1/2
HLA-B*5801 ($n = 1$)	VAALFFFDI	−1.82	822.6	1/1

(i) HLA class I alleles listed in column 1 are those carried by HCMV seropositive donors tested. In parentesis = number of donors bearing the allele
(ii) Prediction based on IEDB score and MHC affinity
(iii) The IEDB score combines proteasome cleavage and peptide processing, TAP transport and MHC binding
(iv) units of IC_{50}[nM]: MHC high affinity (<50 nM); MHC intermediate affinity (<500 nM); MHC low affinity (<5000nM).
Cut-off for selecting HLA-peptide specificities = 5000 nM.
(v) IFN-γ gene expression >2-fold the baseline (no peptide induction). For responding peptides potentially restricted by more than one HLA allele for individual donors, HLA restriction has been assigned according to the highest predicted MHC affinity.
In bold = immunodominant peptides (75% of positive responses in six or more individuals). HLA-A*2402/QYD association was used as internal positive control

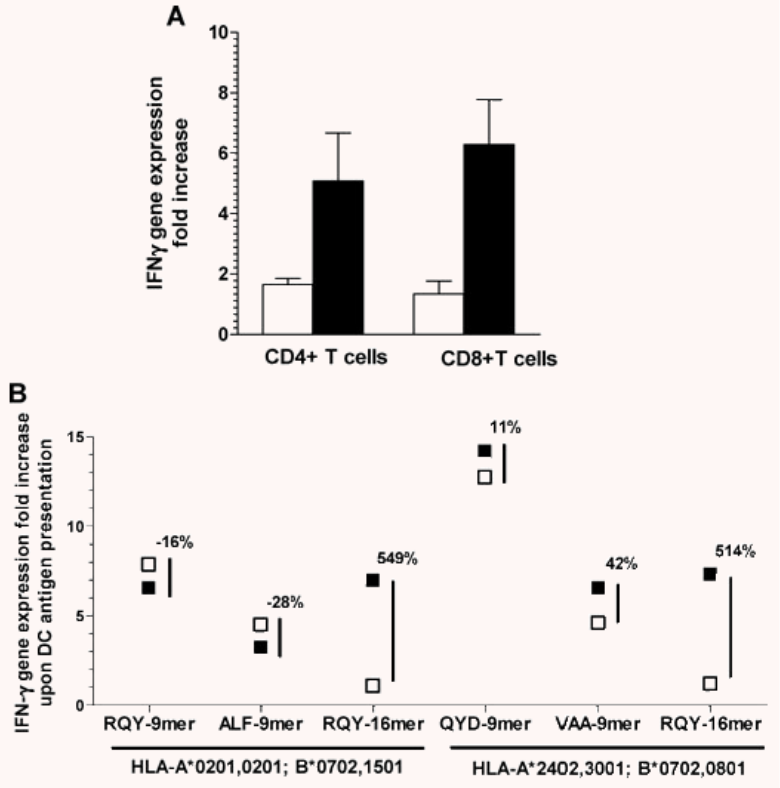

Fig. 3 Glutaraldehyde (Gla)-fixation of dendritic cells jeopardizes the induction of RQY-16mer peptide specific immune responses. (**A**) IFN-γ gene expression by cultured $CD4^+$ and $CD8^+$ T cells is significantly ($P < 0.05$) impaired if RQY-16mer peptide is presented by autologous Gla-fixed (white bars) iDCs, as compared to RQY-16mer treated live iDCs (black bars). Data are reported as IFN-γ gene expression as related to the response in the absence of antigenic peptide. A two-tailed paired t-test was used to define *P*-values ($P = 0.05$; CI 95%). (**B**) Gla-fixation of iDCs (white squares) does not impair responsiveness of $CD8^+$ T cells to HLA class I restricted 9mer-peptides, as compared to reactivity upon peptide presentation by live iDCs (black squares). Instead, a significant increment of IFN-γ gene expression is observed in $CD8^+$ T cells co-cultured with live iDCs in the presence of RQY-16mer peptide, as compared to Gla-fixed cells (514% for the first donor and 549% for the second donor).

presented in the context of matching HLA class I determinants. To test this hypothesis, iDCs from three HCMV-seropositive donors bearing different HLA antigens were glutharaldheyde-fixed and used to stimulate T-cell responses in the presence of RQY-16mer or selected 9mer-peptides. As control, live iDCs were used. We found that the ability of *in vitro* expanded $CD4^+$ or $CD8^+$ T cells to respond to RQY-16mer peptide stimulation by IFN-γ gene expression was significantly jeopardized when fixed iDCs were used as

antigen presenting cells, as compared to live cells ($CD4^+$/IFN-γ: 1.65 ± 0.21 *versus* 5.08 ± 1.58-fold increase, $P = 0.048$; $CD8^+$/IFN-γ: 1.34 ± 0.25 *versus* 6.28 ± 1.47-fold increase, $P = 0.046$; Fig. 3A). In contrast, fixed iDCs fully retained their capability to activate $CD8^+$ T cells in the presence of 9mer-peptides, comparably to their unfixed counterparts (Fig. 3B) [32].

Cytokine production by RQY-16mer specific $CD4^+$ T cells

Due to the active role possibly played by $CD4^+$ T cells in controlling viral infections and considering the high immunogenicity of the RQY-16mer, the activity of specific $CD4^+$ T cells was also tested at protein level. Intracellular cytokine staining (ICS) was performed in $CD4^+$ T cells cultured for 2 weeks in the presence or absence of antigenic peptide and reactivated for 6 hrs by RQY-16mer loaded autologous mDCs. Seven HCMV-seropositive donors were selected to represent 90% the HLA-DRB1* specificities detectable in our group of donors (Table 1A). The two HCMV-seronegative donors were also included as negative controls.

Cut-off values (0.18%) were calculated based on the stainings observed in cultures conducted for 2 weeks in the presence of mDCs and IL-2 but without antigenic stimulation. Percentages of IFN-γ, TNF-α and IL-2 positive cells in 6-hr assays performed in the presence of antigenic peptide significantly exceeding cut-off values were observed in antigen pre-stimulated cultures from each HCMV-seropositive donor tested irrespective of their specific HLA DRB1* association (IFN-γ: 4.76 ± 10.18%, median 0.84%, range 0.24–27.8%; TNF-α: 5.69 ± 13.02, median 0.95%, range 0.22–35.2%; IL-2: 3.11 ± 7.14, median 0.39%, range 0.23–19.3%, Fig. 4A). In contrast, percentages of ICS cytokine positive cells in 6-hr assays performed in the absence of antigenic peptide, were equal to or lower than cut-off values (0.18 ± 0.03% for IFN-γ, 0.08 ± 0.04% for TNF-α and 0.08 ± 0.04% for IL-2). Notably, RQY-16mer stimulation of cells from HCMV-seronegative donors did not result in the production of cytokines (<0.05% in all cases). It is of note that analysis of RQY-16mer by NetMHCIIpan algorithm revealed the existence within this peptide of a number of core sequences capable of binding the DRB1* allelic products expressed by the donors under investigation, as reported in Fig. 4B.

Functional features of RQY-16mer expanded $CD4^+$ T cells

To confirm the ability of RQY-16mer peptide to activate $CD4^+$ T cells and to demonstrate their possible direct role in the control of HCMV infection, we investigated their antigen-specific proliferation and cytotoxic activity.

Freshly obtained $CD4^+$ T cells from HCMV-seropositive donors ($n = 4$) accounting for a high percentage of the HLA-DRB1* alleles expressed in our cohort (75%) were co-cultured with autologous mDCs in the presence or absence of the specific peptide for 7 days. A 0.5 μg/ml concentration of RQY-16mer sufficed to stimulate a significant proliferation of specific $CD4^+$ T cells in all donors tested, thus confirming its ability to reactivate and expand immune responses from HCMV-seropositive individual irrespective of their HLA-DRB1* specificities. It is remarkable that core sequences within RQY-16mer capable of binding each of these class II determinants were revealed by analysis with NetMHCIIpan algorithm. In contrast no response was induced upon stimulation of cells from a control HCMV-seronegative donor DRB1*07,14 ($P = 0.4$) (Fig. 5A).

The cytotoxic capacity of RQY-16mer peptide stimulated $CD4^+$ T cells was then evaluated by caspase release assays. Immature DCs were infected with HCMV as previously described [33]. $CD4^+$ T cells from HCMV-seropositive donors bearing different HLA class II antigens ($n = 3$) were expanded *in vitro* for 2 weeks by RQY-16mer peptide stimulation. They were then incubated for 4 hrs with HCMV-infected iDCs at 10:1 E/T ratio. Cultures were then harvested and stained for intracellular caspase-3 release and for surface expression of CD1a as marker for DC identification.

Caspase-3 production in the absence of expanded T cells was negligible in HCMV-infected iDCs (positive cells = 0.02 ± 0.01%, mean fluorescence intensity [MFI] = 3, left histogram, Fig 5B) and comparable to that of untreated iDCs (data not shown). Co-culture in the presence of RQY-16mer peptide expanded $CD4^+$ T cells induced caspase-3 production in mock-infected autologous iDC targets, likely due to unspecific stimulation of cytototoxic activity by the IL-2 used in the T-cell expansion protocol, and, possibly, at least in part, to their expression of endogenous HCMV genes, including pp65 [34] (positive cells = 19.72 + 7.56%, MFI = 57, mid histogram, Fig. 5B). Superinfection of target cells with HCMV significantly increased their sensitivity to the cytotoxic activity of RQY-16mer stimulated $CD4^+$ T, resulting in a 60% MFI increase upon caspase-3 staining (positive cells = 59.55 + 4.01%, MFI = 280, right histogram, Fig. 5B). Figure 5C reports cumulative data obtained in three independent experiments (mock-infected iDCs fluorescence index [FI] = 3.33 ± 1.52 *versus* HCMV-infected iDCs FI = 77.26 ± 7.39, $P = 0.0073$).

IFN-γ an IL-2 protein production upon RQY-16mer peptide stimulation in kidney transplanted patients

HCMV infection is frequently reactivated in pharmacologically immunosuppressed transplanted patients. Thus, the capability of specific antigenic peptides to stimulate immune responsiveness in these conditions is of utmost clinical relevance. We tested the ability of RQY-16mer of reactivating a peptide-specific immune response in isolated T cells from kidney transplanted patients ($n = 10$; Table 3A). All patients were treated with an immunosuppressive regimen consisting of mycophenolate, cyclosporine and prednisone and tested 183 ± 102 (range 83–374) days after transplantation. One HCMV-seronegative patient (P3) transplanted

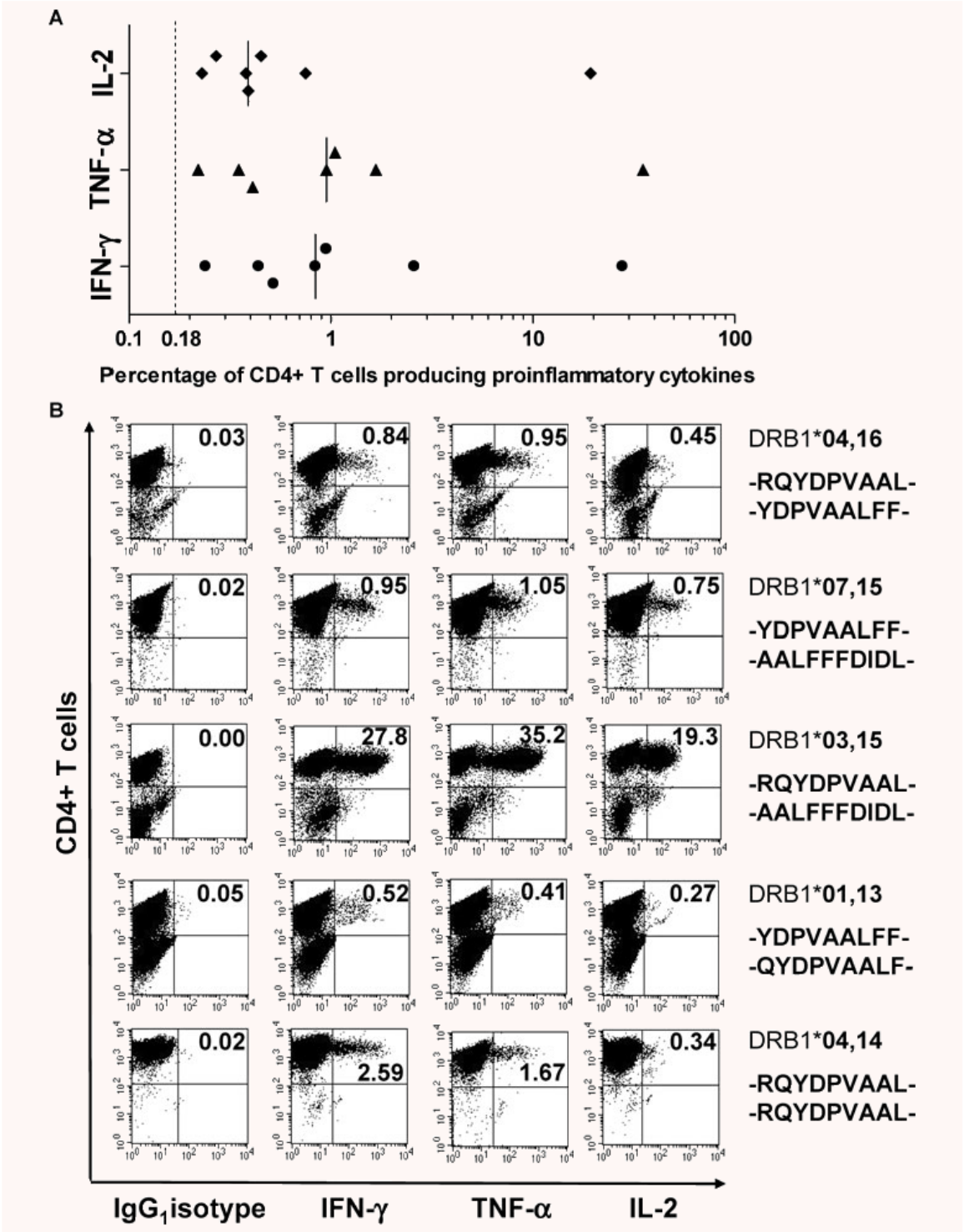

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Fig. 4 RQY-16mer is a promiscuous epitope stimulating cytokine production by $CD4^+$ T cells across a wide range of HLA class II restrictions. (**A**) Percentages of $CD4^+$ T cells producing pro-inflammatory cytokines (IFN-γ: circles; TNF-α: triangles; IL-2: diamonds) upon RQY-16mer induction were assessed by ICS in cells from seven donors expressing 90% of the HLA-DRB1* alleles represented in our group. Previously cultured for 2 weeks in the presence of the peptide, as detailed in the 'Materials and methods' section. Each symbol represents the average of two independent experiments performed by using cells from the same donor. Cut-off limit was set at 0.18% (dotted line) based on RQY-16mer peptide-induced cytokine production by $CD4^+$ T cells expanded for 2 weeks in the presence of mDC and IL-2, but in the absence of peptide. Quadrants were set based on an IgG_1 isotype control. Median percentages of responding cells are indicated by bars. (**B**) Representative ICS of RQY-16mer stimulated cytokine production from HCMV-seropositive donors. Data reported in the quadrants refer to percentages of $CD4^+$ T cells showing evidence of the production of the indicated cytokines induced by RQY-16mer in $CD4^+$ T cells from different donors expanded for 2 weeks in the presence of the peptide. For each donor, HLA-DRB1* tissue typing data and predicted netMHCIIpan algorithm associations of core sequences embedded within RQY-16mer with defined allelic products are also reported (characters in bold, right column).

Fig. 5 Functional features of RQY-16mer induced $CD4^+$ T cells. (**A**) Proliferation of $CD4^+$ T cells upon RQY-16mer stimulation. Freshly isolated PBMCs from four HCMV seropositive donors and one seronegative donor were cultured for 1 week in the presence (black bars) or absence (white bars) of RQY-16mer, as detailed in material and methods. Proliferation was measured by ^{3}H-Thymidine incorporation. Results are reported as mean of cpm ± S.D. of triplicate wells. Two-tailed paired t-test (stimulation *versus* non-stimulation) was used to define *P*-values ($P = 0.05$; CI 95%). Significant increases in RQY-16mer induced proliferation were observed in seropositive donors (DRB1*04,14 $P = 0.04$; DRB1*01,13 $P = 0.05$; DRB1*04,12 $P = 0.03$; DRB1*03,15 $P = 0.004$), but not in the seronegative donor (DRB1*07,14, $P = 0.4$). (**B**) Caspase-3 production by HCMV-infected iDCs co-cultured with RQY-16mer expanded $CD4^+$ T cells. Following RQY-16mer driven expansion (see 'Materials and methods' section), $CD4^+$ T cells were co-cultured at 10:1 E/T ratio with autologous iDCs infected at a MOI of 10 with HCMV VR1814 or mock infected. Caspase-3 production, as detected in CD1a+ DCs was used as read-out. The histogram shows a significant increase of caspase-3 production in either mock-infected (middle red peak) or HCMV-infected iDCs (right peak), upon co-culture with RQY-16mer expanded $CD4^+$ T cells, as compared to that detectable in negative control iDCs in the absence of effectors (left peak). A 60% increase of caspase-3 production, as detectable by specific mean fluorescence intensity (MFI), in HCMV-infected iDCs as compared to mock-infected counterparts was observed in the presence of RQY-16mer expanded $CD4^+$ T cells. (**C**) Caspase-3 production by HCMV infected *versus* mock-infected iDCs following co-culture with $CD4^+$ T cells expanded in the presence of RQY-16mer, as observed in three independent experiments by using cells from different HCMV seropositive donors. The fluorescence index was calculated by the following formula: (MFI experimental sample − MFI background)/(MFI background)%. Statistical significance ($P = 0.0073$) was analysed by two-tailed paired t-test.

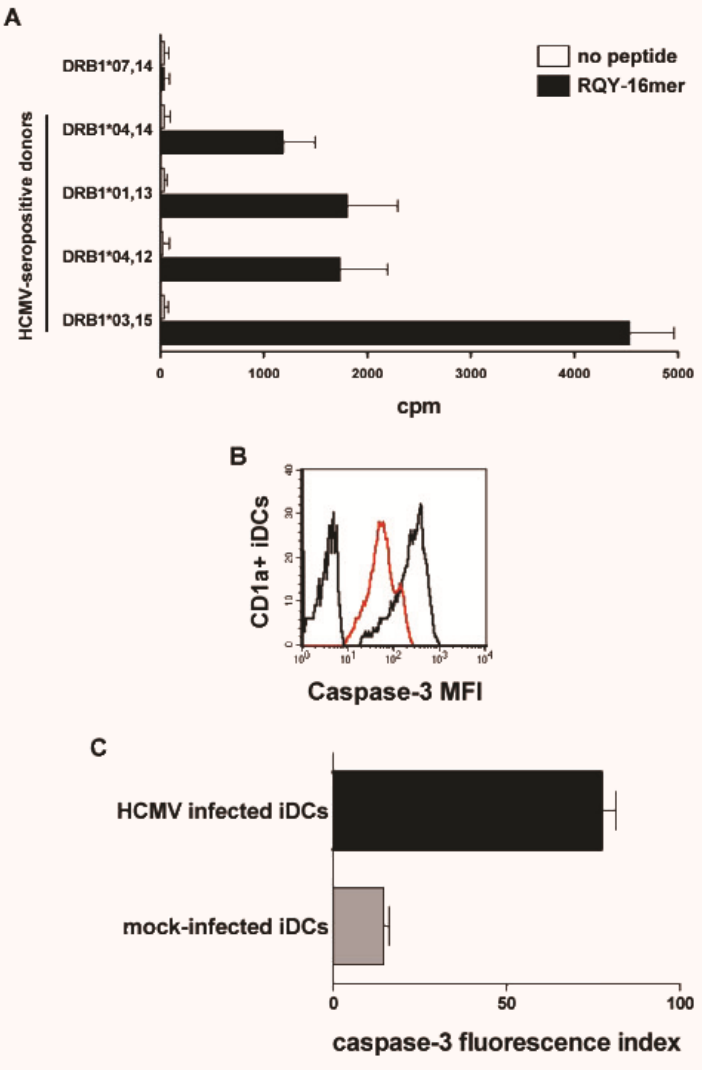

Table 3 Effect of RQY-16mer peptide stimulation in pharmacologically immunosuppressed kidney transplanted patients

A

	Full typing								RQY-16mer expanded $CD4^+$ T cells (iii)	
Code	HLA-A*	HLA-B*	HLA-DRB1*	Donor (D) Recipient (R) serology	Infection type	viral replication (i)	antiviral prophylaxis	day of the test post transplant	IFN-γ	IL-2
P1	1101,3101	35,5101	11,14	D-/R+	reactivation	none	-	154	++	++
P2	0101,3001	0801,4202	17,18	D+/R+	reactivation	none	-	254	++	++
P3	0301,2402	0702, 0702	4,15	D+/R−	primary	(325cp/ml)	Valganciclovir (ii)	85	−	−
P4	0101,0101	0801,5701	07,17	D+/R+	reactivation	(488cp/ml)	−	320	−	−
P5	0101,2301	4401,52	07,08	D−/R−	none	none	−	203	−	−
P6	02,2402	46,56	09,14	D+/R+	reactivation	none	−	162	−	−
P7	1101,1201	0501, 52	01,15	D−/R+	none	none	−	91	+++	+++
P8	1101,1101	0801,55	12,14	D−/R+	reactivation	(1045cp/ml)	−	83	−	+
P9	0201,1101	35,53	01,15	D−/R+	none	none	−	112	−	−
P10	2402,6801	4402,62	04,17	D+/R+	none	none	−	374	+++	++

(i) EDTA anti-coagulated whole blood. Limit of detection 300 cp/ml
(ii) 450 mg daily/3 months
(iii) cytokine protein production (ICS) fold increase above unstimulated cells (CI 95%; $P < 0.05$).
(+) =2fold; (++) > 2 < 3fold; (+++) >3fold.

B

Code	HLA-A*	HLA-B*	9-mer peptide specific induction upon RQY-16mer *in vitro* expanded $CD8^+$ T cell (iv)							
			RQY	QYD	DPV	VAA	ALF			
P4	0101,0101	0801,5701		+	+					
P6	02,2402	46,56		+++						
P9	0201,1101	35,53	+++	−	+++	++	++			
P10	2402,6801	4402,62		+++	+	−				

(iv) IFN-γ protein production (ICS) fold increase above unstimulated cells (CI 95%; $P < 0.05$).

(+) =2-fold; (++) > 2 < 3fold; (+++) >3-fold.

with a kidney from a HCMV-seropositive donor and thereby at high risk of HCMV infection underwent a prophylactic regimen (VALgcv) for the first three months after transplant.

T cells from these patients were cultured in the presence of antigenic peptide for 2 weeks and then tested by ICS as detailed in the 'Materials and methods' section. IFN-γ and IL-2 protein production upon 6 hrs RQY-16mer stimulation was significantly increased in comparison with unstimulated cells in $CD4^+$ T lymphocytes from four (P1,2,7,10) and five (P1,2,7,8,10) of the eight HCMV-seropositive patients, respectively. However, no responses were observed in either patient P3 undergoing primary infection under antiviral prophylactic treatment or in the HCMV-seronegative

patient P5 (Table 3A). Responsiveness to class I restricted epitopes encompassed by RQY-16mer was also investigated in $CD8^+$ T cells from four patients. Interestingly, in three of them, P4, P6 and P9, showing no $CD4^+$ T-cell responsiveness, 9mer peptides nested within RQY-16mer were able to elicit IFN-γ production in $CD8^+$ T cells. In P10 $CD8^+$ T-cell responsiveness to nested 9mers matched the vigorous response of $CD4^+$ lymphocytes to RQY-16mer (Table 3B).

Discussion

A number of different approaches are currently being investigated to prevent HCMV infection or its reactivation. They include active immunization with live attenuated virus, subunit vaccines, recombinant viral vectors, neutralizing immunoglobulins, synthetic peptides or DNA vaccines [35]. Furthermore, adoptive transfer of HCMV-specific T cells expanded by using specific and unspecific HLA matching peptides has also been utilized [36].

In this study we addressed the identification of HCMV pp65-derived antigenic peptides of potential use across a wide range of HLA specificities. Given the decisive role of helper T cells in the optimal expansion of CTL, and, in particular, in the anti-HCMV-specific immune response, the characterization of both class I and II restricted epitopes was envisaged. Clearly, single peptides gathering the ability to stimulate HLA class I and II restricted T cells beyond the limits of foretold MHC-peptide binding would represent reagents of potentially high clinical relevance.

Algorithms are commonly used to predict HLA-peptide affinity and they usually provide preliminary insights for the identification of novel antigenic epitopes [26]. Binding to HLA gene products represents a fundamental prerequisite of peptide immunogenicity [37–39]. However, studies based on linear regression analysis [40, 41] have demonstrated that the immunogenicity of a number of peptides predicted to avidly bind HLA-restricted molecules is not always validated by *in vitro* or *in vivo* studies.

In this work we demonstrate that concurrent use of different epitope prediction algorithms is not only highly informative regarding the likelihood of individual peptides to be processed and presented within specific HLA restrictions but it is also able to predict with low margin of error the immunogenic potential of specific sequences.

The novel HCMV-derived immunogenic peptide described here is characterized by a potentially high clinical relevance due to a number of important features.

First, RQY-16mer is able to stimulate HCMV pp65 specific responses in both $CD4^+$ and $CD8^+$ T cells, restricted by a wide range of HLA class I and II determinants. This responsiveness is not related to intrinsic stimulatory activity because it does not extend to cord blood lymphocytes. Thus, a single reagent might qualify as synthetic immunogen for potentially large populations exposed to HCMV infection or reactivation. A large number of pp65-derived class I restricted antigenic peptides have been described [16] and key-target class II restricted peptides have also been identified [42]. However, the number of peptides capable of simultaneously stimulating $CD4^+$ and $CD8^+$ T cells is very limited [43]. Notably, the latter reagents usually induce CTL responses restricted by one HLA class I determinant only. Although they have provided proof of principle of the possibility of activating $CD8^+$ T cells by using 'long' peptides, they hardly qualify for a wider clinical use.

Interestingly, RQY-16mer displays a relatively hydrophobic pattern and its capability to stimulate either $CD4^+$ or $CD8^+$ T-cell responsiveness requires processing by live antigen presenting cells. These data suggest that its promiscuity could be ascribed to internalization into iDCs, peptide trimming by immunoproteasome activity and processing of shorter sequences swapped into different HLA class I and II clefts to better fit multiple associations, as predicted by algorithm binding scores for RQY-16mer relevant peptide-cores. The sequence might thus retain the power of inducing specific immune responses restricted by a wide range of HLA allelic products.

Intriguingly, RQY-16mer has previously been studied by other groups [30, 43], but its high immunogenicity was not fully disclosed. It is worth noting that in these studies either total PBMC were used as responder cells [30, 44] or T-cell stimulation was achieved by using TNF-α matured DC or transfected B cells as APC [43, 45]. Dendritic cells matured through TLR-4 triggering used in our study are characterized by a significantly higher APC capacity. In particular, they produce IL-12, while TNF-α matured DCs fail to do so [46].

A second important feature of the RQY-16mer is represented by its capability to induce an unusually wide range of effector functions in $CD4^+$ T cells. As expectable, specific $CD4^+$ T cells are capable of producing IFN-γ and TNF-α and of proliferating in response to RQY-16mer. Most interestingly, however, they are also able to elicit a significant cytotoxic activity against virus-infected autologous target cells. $CD4^+$ HCMV-specific CTLs have been typically detected during early phases of HCMV infection [47]. However, their elicitation upon *in vitro* stimulation by antigenic peptides represents a relatively infrequent event [43].

Third, and most importantly, the RQY-16mer is able to stimulate $CD4^+$ and $CD8^+$ T-cell responses in pharmacologically immunosuppressed patients. These data suggest that this reagent could be advantageously utilized to prevent or treat HCMV infection or reactivation in a population of patients at high risk of developing disease.

Finally, we are confident that the concurrent use of different predictive algorithms, exemplified in this study, could be of particular relevance for the identification of immunogenic sequences within candidate viral and tumour antigens, irrespective of discrete MHC associations [48–51]. Universal peptides eventually encapsulated into virosome formulations [52, 53] or encoded by viral vectors [52, 54] might represent key-reagents for the generation of powerful vaccine preparations aimed at strengthening cellular immune responses. Databases inclusive of their sequences might help unravelling specific motives associated with the ability to promiscuously activate T cells across a wide variety of MHC class I and II restricting determinants.

Acknowledgements

This work was partially supported by a grant from Swiss National Funds for Scientific Research to Giulio C Spagnoli. Cell-free endotheliotrophic (HUVEC) HCMV VR1814 strain and the goat anti-IE72 mAb were provided by G. Gerna (Policlinico San Matteo, Pavia, Italy). Recombinant human IL-4 and IL-6 were a gift of A. Lanzavecchia (Institute for Research in Biomedicine, Bellinzona, Switzerland). Recombinant human GM-CSF was a gift from the Laboratorio Pablo Cassara', Buenos Aires, Argentina.

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3.3. Differential patterns of Large Tumor Antigen specific immune responsiveness in patients with BK polyomavirus positive prostate cancer or benign prostatic hyperplasia.

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JVI Accepts, published online ahead of print on 30 May 2012
J. Virol. doi:10.1128/JVI.00005-12

Differential patterns of Large Tumor Antigen specific immune responsiveness in patients with BK polyomavirus positive prostate cancer or benign prostatic hyperplasia

Running title: BKV L-Tag specific immune response in PCa

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Abstract word count: 230

Text word count: 5214

ABSTRACT

The role of the polyomavirus BK (BKV) large tumor antigen (L-Tag) as target of immune response in patients with prostate cancer (PCa) has not been investigated so far. In this study we have comparatively analyzed humoral and cellular L-Tag specific responsiveness in age matched patients bearing PCa or benign prostatic hyperplasia (BPH), expressing or not expressing BKV L-Tag specific sequences in their tissue specimens, and in non-age-matched healthy individuals. Furthermore, results from patients with PCa were correlated to 5-year follow-up clinical data focusing on evidence of biochemical recurrence (BR) following surgery (PSA≥0.2ng/ml).

In peripheral blood mononuclear cells (PBMC) from patients with PCa with evidence of BR and BKV L-Tag positive tumors, stimulation with peptides derived from BKV L-Tag, but not those derived from Epstein Barr virus, influenza virus or Cytomegalovirus, induced a peculiar cytokine gene expression profile, characterized by high expression of IL-10 and TGFβ-1 and a low expression of IFN-γ genes. This pattern was confirmed by protein secretion data and correlated with high levels of anti BKV L-Tag IgG. Furthermore, in PBMC from these PCa bearing patients, L-Tag derived peptides significantly expanded an IL-10-secreting $CD4^{+}CD25^{+(high)}CD127^{-(dim)}FoxP3^{+}$ T cell population with an effector memory phenotype ($CD103^{+}$) capable of inhibiting proliferation of autologous anti-CD3/CD28 triggered $CD4^{+}CD25^{-}$ T cells. Collectively, our findings indicate that potentially tolerogenic features of L-Tag specific immune response are significantly associated with tumor progression in patients with BKV+ PCa.

KEY WORDS

Human Polyomavirus BK Large Tumor Antigen, Prostate Cancer, Regulatory T cells, Viral Immunology.

INTRODUCTION

Prostate cancer (PCa) represents the first leading cause of cancer morbidity and the third of cancer death in men in developed countries with a worldwide incidence rate of 14% of total newly diagnosed malignancies and a worldwide total cancer mortality rate of 6% (29). A contemporary model of PCa induction and progression should include the analysis of the contribution of inflammation to the development of preneoplastic or neoplastic lesions (24). Indeed, proliferative inflammatory atrophy (PIA) of the prostate has recently gained importance as potential precursor of prostatic intraepithelial neoplasia (PIN) and overt PCa (15), (14). This owes particularly to the prevalence of PIA in the peripheral zone of the organ, where histological transition between PIA and PIN usually occurs (42).

The low rate of mutations detected in tumor suppressor genes pRB1 and p53 in primary PCa cells (16) has suggested a possible role of inflammatory agents ubiquitous in the urinary tract with unique oncogenic functions, that is of sequestering wild-type products of tumor suppressor genes. Polyomaviruses' main regulatory proteins large tumor antigens (L-Tag) interfere with wt-p53 binding to cellular DNA during virus infection (4), thereby impairing p53 control on cell growth activity and possibly leading to oncogenic transformation in non-permissive cells (6). As such, the human urotheliotrophic polyomavirus BK (BKV) has been suggested to prominently associate with the development of cancer (26, 27), and, in particular, of urinary tract malignancies (3, 12, 54).

There is a continuing debate on BKV expression in overt cancers (1, 5). However, the possibility of "hit and run" carcinogenic mechanisms induced by BKV cannot be excluded (13). Genetically rearranged BKV variants (45), (22), presumably difficult to detect by commonly used assays, might exist in the urinary tract and be responsible for neoplastic transformation in prostate

cells (i.e. URO-1) (37). Therefore, BKV has been indicated as potential co-factor in the earliest stages of PCa (13).

Detection and expression of BKV L-Tag sequences in preneoplastic prostate tissues (12) have prompted us to investigate the role of this viral antigen as target of immune response. Thus, in this study we have addressed humoral and cellular responsiveness to BKV L-Tag in patients with benign prostatic hyperplasia (BPH) or newly diagnosed PCa and we have correlated viral and immunological features with PCa status.

PATIENTS & METHODS

Patients and clinical follow-up

One hundred ten consecutive patients diagnosed for either prostate cancer (PCa, n=60; PSA>4ng/ml and suspicious digital rectal examination (DRE) and/or positive for early diagnosed high grade prostate intraepithelial neoplasm (HGPIN) at biopsy) or benign prostate hyperplasia (BPH n=50; urinary obstructive symptoms and acute urinary retention according to the International Prostate Symptom Score, IPSS) were enrolled in the study at the Department of Urology of the University Hospital of Basel, Switzerland, and at the Division of Urology of the University Hospital of Zurich, Switzerland, upon informed consent, following approval by Cantonal Ethical Committees of Basel and Zurich. Five-year follow-up after surgery was completed for 48/60 PCa patients (80%). Timing of biochemical recurrence (BR+), with early censoring if only 1 or 2 values were available, was established at the first ≥0.2ng/ml PSA detection. BR- patients were those showing clearly negative PSA values (<0.04ng/ml) during complete follow-up. Data were collected according to the American Society for Therapeutic Radiation and Oncology (ASTRO) criteria (23), considering both ACTUARIAL and ASTRO-time analytical methods, since ASTRO-censoring allowed to define biochemical failure by 3 consecutive increases in PSA values in 3/48 PCa only.

Virus detection in tissue specimens

Specimens from tissues excised during surgical procedures were formalin-fixed and paraffin-embedded. DNA was extracted from 3 sections (thickness, 5 µm) randomly picked within the tumor area (PCa) or within the atrophic-hyperplastic gland (BPH) using the QIAamp DNA Mini Kit (Qiagen, Basel, Switzerland), according to the manufacturer's instructions.

antigen (691 amino acids, Swiss-Prot P14999) and tiled at 11 amino acid pace. Similarly, the negative control PepMix HIVgag peptide-pool (123 peptides including B gag motif) was also provided by JPT. As positive control, the PepMix CEF (Cytomegalovirus, Epstein Barr virus and Influenza virus) pool of 23 8-11mer peptides recognized by CD8 positive T cells and presented by 11 class I HLA-A and HLA-B alleles (11) was used (JPT, Berlin Germany). For *in vitro* expansion and proliferation assays, both HIV peptide-pool (see above) and the Human Cytomegalovirus promiscuous epitope $pp65_{340\text{-}355}$ (41) (Princeton Biomolecules, Langhorne, PA, USA) were used as controls.

"*Ex vivo*" induction and detection of cytokine gene expression by qRT-PCR

Peripheral blood mononuclear cells (PBMC) isolated from venous blood by Ficoll-Hypaque density gradient centrifugation were resuspended in RPMI medium supplemented with 100 µg/ml Kanamycin, 10 mM Hepes, 1mM sodium pyruvate, 1 mM Glutamax and nonessential amino acids (all from GIBCO Paisley, Scotland) (complete medium) and 5% human serum (BlutspendezentrumUniversitätsSpital Basel, Switzerland), at a final concentration of 1×10^6 cells/ml, plated in 96 U-bottom well plates (200 µl/well) and incubated for overnight resting. Cells were then stimulated with either test (L-Tag) or control (CEF, HIV) peptide-pool (1 µg/ml) or PHA (1 µg/ml) and harvested after 3 hours for RNA extraction (RNeasy® Mini Kit Protocol, Qiagen, Basel, Switzerland) and cDNA synthesis (Invitrogen, Carlsbad, CA, USA). Quantitative gene amplification (qRT-PCR) was performed, as previously described (40), by an ABI prism™ 7500 FAST sequence detection system using TaqMan® Universal PCR Master Mix Reagents Kit and "on demand" sets of primers and probes for cytokine gene expression (IFN-γ, IL-10, TGF-

β1) (Applied Biosystems, Rotkreuz, Switzerland). β-actin was used as endogenous reference gene and normalized data were analyzed by the $2^{-\Delta\Delta Ct}$ method (34).

Cell cultures

Monocytes ($CD14^+$) sorted from PBMC by magnetic beads (Miltenyi Biotech) were cultured from 5 to 7 days in RPMI complete medium supplemented with 10% FCS (GIBCO Paisley, Scotland), 0.004% β-mercaptoethanol, rhIL-4 (1000 U/ml) and rhGM-CSF (50 ng/ml) to generate immature DCs (iDCs). To induce maturation, iDCs were overnight exposed to 1 µg/ml LPS (Abortus Aequi, Sigma-Aldrich, St. Louis, MO, USA). $CD4^+$ T cells positively sorted from PBMC by magnetic beads were cultured in complete medium with 5% human serum in 24 flat-bottom well plates at a $1x10^6$ cells/ml concentration in the presence of autologous irradiated mature DC (mDCs), previously pulsed for 2 hours with peptide pools (10 µg/ml), either for priming (day 0) or re-stimulation (day 7). Recombinant human IL-2 (rhIL-2; Hoffmann-LaRoche, Basel, Switzerland) was added to cultures at 1ng/ml, 1ng/ml and 5ng/ml, on days 3, 7 and 10, respectively.

Regulatory T cell cultures were established by *ex vivo* magnetic sorting of $CD4^+C25^+CD127^{-/dim}$ subset, as previously described (48). Cells were subsequently stimulated with L-Tag peptide-pool (5µg/ml) or control peptides, including $HCMVpp65_{340\text{-}355}$ and HIV peptide-pool, in the presence of 10 µg/ml anti-CD28 (BD Bioscience, Allschwil, Switzerland) and 5 ng/ml of rIL-2. Cultures were similarly restimulated on day 7. IL-10-secreting Treg were sorted on day 14 by cytokine secretion assay (Miltenyi Biotec, Germany). For suppression assays, $CD4^+CD25^{+(high)}$ $CD127^{-(dim)}$, IL-10-secreting T cells were cultured in the upper chamber of 24-transwell plates (BD Bioscience, Allschwil, Switzerland) with a double amount

(ratio=0.5:1) of autologous $CD4^+CD25^-$ T cells, stimulated with 1mg/ml of anti CD3/CD28 (BD Bioscience, Allschwil, Switzerland) seeded in the lower chamber, and cultured for 8 days. $CD4^+CD25^-$ T cells proliferation was measured using Cell Proliferation Reagent WST-1 (Roche Applied Science, Rotkreutz, Switzerland) according to manufacturer's instructions and absorbance was read at 450nm with an ELISA plate reader.

Regulatory T cell quantification by flow cytometry

$CD4^+$ T cells stimulated by peptide-pools, as described above, were stained with anti-CD4 FITC and anti-CD25 APC antibodies (BD Biosciences, Allschwil, Switzerland). Anti FoxP3 PE antibodies and FoxP3 Fix/Perm buffer were used for intracellular staining according to manufacturer's protocol (eBioscience, Vienna, Austria). Separation into CD25 bright ($CD25^{high}$) and CD25 dim ($CD25^{low}$) cells was carried out using a fluorescence cut-off defined in healthy donors (HD). Anti-CD103 PerCP (eBioscience, Vienna, Austria) and anti-IL-10 APC intracellular staining (BD Bioscience, Allschwil, Switzerland) were additionally carried out for the identification of IL-10-secreting activated Treg. Data were acquired on a FACSCalibur flow-cytometer equipped with Cellquest software (Becton Dickinson, San Jose`, CA, USA).

Cytokine measurement

The FlowCytomix Simple kit (eBioscience, Vienna, Austria) was used to measure IFN-γ, IL-10 and TGF-β1 protein release in culture supernatants of peptide-stimulated T cells. Samples were run on a FACSCalibur flow-cytometer equipped with Cellquest software (Becton Dickinson, San Jose`, CA, USA), analyzed with BMS FlowCytomix software (eBioscience, Vienna, Austria), and referred to a standard curve for quantification.

Statistical analysis

Statistical analysis was performed with Graph Pad Prism (version 5.1) and SAS/STAT (version 9.1). Data were reported as mean ± standard deviations (SD) or mean ± standard errors (SE), median and ranges where appropriate. Distributions of categorical markers were analyzed by both χ^2 and Fisher's exact tests. Groups following normal distribution (Shapiro-Wilk test) were compared with *t*-tests (either Pooled or Cochran method) and *F*-test was used to calculate variance among groups of samples. If non-normal distribution was indicated, nonparametric tests such as Mann-Whitney U tests and Spearman's ρ correlation analyses were used. *P* values <0.05 (CI 95%) were considered statistically significant.

RESULTS

Clinical profiles of patients

The average age of patients enrolled in the study (n=110) was 64±7.7 years (range 49-90) for PCa (n=60) and 67±7.4 years (range 48-81) for age-matched BPH (n=50), respectively.

Out of 60 PCa patients, 1 was diagnosed with clinical stage pT1b, 10 with pT2a, 2 with pT2b, the majority (n=30; 51%) with clinical stage pT2c, 4 with pT3a and 3 with pT3b. Ten (17%) patients had tumors whose stage at diagnosis could not be assessed. Average serum prostate specific antigen (PSA) value was 17.4±36.2 ng/ml (median: 6.7 ng/ml, range: 1.6-206 ng/ml) in PCa patients before surgery (n=50, 83%) and 5.6±5.7 ng/ml (median: 3.9 ng/ml, range: 0.4-26 ng/ml) in BPH patients (n=36, 72%). Gleason Score (GS) of tumor specimens (n=55, 92%) ranged from 5 to 9 according to immunohistochemical analysis (Table 1).

L-Tag DNA detection in tissue specimens and BKV specific humoral response

Presence of polyomavirus BK was analyzed by BKV L-Tag DNA detection in surgically excised PCa (n=43/60, 72%) and BPH (n=38/50, 76%) specimens. Positive samples (hereafter referred to as BKV+) were identified among both PCa (mean=451.4 copies/10^5cells; n=18/43, 42%) and BPH (mean=391.5 copies/10^5cells; n=12/38, 32%) with no significant quantitative differences among groups of patients (p=0.72) (Figure 1a). No significant association (χ^2) with cancer (p=0.18) or Gleason Score (GS+ ≥7; p=0.82) was observed. However, high PSA levels (PSA ≥4ng/ml) were more frequently detectable in sera from patients with BKV+ PCa ($p<0.05$).

Humoral response against BKV VP1- and L-Tag was analyzed in sera from patients with PCa and BPH. Upon sample stratification based on BKV L-Tag DNA tissue testing, we noted that levels of L-Tag specific IgG (OD) were significantly higher in patients bearing BKV+ than BKV-

PCa (p=0.01), while they were similarly low in patients with BKV+ or BKV- BPH. In contrast, anti VP1 IgG levels were similar in patients with PCa or BPH, irrespective of their BKV molecular status (Figure 1b). Overall, detectable evidence of humoral responsiveness to L-Tag (OD≥0.04) was observed in 17/18 (94%) patients with BKV+ PCa and in 7/12 (58%) patients with BKV+ BPH with a significant association (χ^2) between BKV+ lesions and L-Tag IgG only in patients with PCa (p=0.005) (Table 1).

Cytokine gene expression pattern in PBMC from patients with PCa or BPH upon *ex vivo* BKV L-Tag peptide-pool stimulation

Humoral response data, showing a high level of L-Tag IgG response in patients bearing BKV+ PCa, prompted us to explore cellular immune responsiveness to this antigen. Freshly isolated PBMC from 60 patients with PCa and 50 age-matched patients with BPH were 3-hour *ex vivo* stimulated with an L-Tag peptide-pool and IFN-γ and IL-10 cytokine gene expression were used as readout. A HIV peptide-pool with B gag motif was used as negative control to provide a baseline allowing the calculation of cytokine gene expression fold changes.

In L-Tag IgG seronegative patients (PCa n=14, BPH n=12, Table 1), regardless of underlying disease, L-Tag peptide-pool stimulated cytokine gene expression was usually low (Figure 2a). However, in L-Tag IgG+ patients with PCa (n=46), L-Tag peptide-pool induced a significantly higher IL-10 gene expression, (p=0.03), but rather a decreased IFN-γ gene expression (p=0.04) as compared to seronegative patients. In contrast, in L-Tag IgG+ patients with BPH (n=38) L-Tag peptide-pool did not induce significantly higher IFN-γ (p=0.8) and IL-10 (p=0.9) gene expression, as compared to seronegative patients. Accordingly, these tests also showed that L-Tag peptide-pool induced a higher IL-10 than IFN-γ gene expression in L-Tag IgG+ patients with

PCa (n=46; p=0.0001) and, to a lesser, non-significant, extent, in L-Tag IgG+ patients with BPH (n=38; p=0.06) (Figure 2a).

To investigate the relationship eventually occurring between BKV L-Tag specific humoral response and L-Tag-specific gene expression pattern, L-Tag specific IgG activity was plotted against IL-10/IFN-γ gene expression ratio. A significant direct correlation was indeed observed in patients with PCa (Spearman: ρ=0.4, p=0.009), but not in patients with BPH (ρ=-0.2, p=0.3) (Figure 2b).

These results urged us to comparatively investigate cytokine gene expression induced by L-Tag peptide-pool, CEF peptide-pool or PHA in L-Tag seropositive patients with PCa or BPH and in seropositive gender but not age-matched healthy donors (HD). Contrary to PBMC from IgG+ patients with PCa and BPH (see above), L-Tag peptide-pool stimulation of PBMC from L-Tag IgG+ HD induced a IFN-γ gene expression significantly higher than IL-10 gene expression (n=8; p=0.003). Most importantly, CEF peptide-pool and PHA induced a similar, significantly higher IFN-γ than IL-10 gene expression in PBMC from patients with PCa or BPH ($p<0.0001$) or from HD ($p<0.001$) (Figure 2c).

Notably, IL-10 gene expression induced by L-Tag peptide-pool was significantly higher than IL-10 gene expression induced by CEF or PHA in PBMC from L-Tag IgG+ patients with PCa ($p<0.0001$ and p=0.0002, respectively) or BPH ($p<0.0001$ and p=0.001, respectively) (Figure 2c). In PBMC from HD, IL-10 gene expression was similarly negligible irrespective of the stimuli used.

Remarkably, IFN-γ gene expression could be induced by CEF of PHA to comparably high extents in PBMC from L-Tag IgG+ patients with PCa or BPH or from HD (Figure 2c), thereby

suggesting a specificity of the cytokine gene expression pattern induced by L-Tag stimulation in PBMC from L-Tag IgG+ patients.

BKV L-Tag peptide-pool stimulated cytokine gene expression pattern in PBMC from patients bearing BKV+ PCa or BPH

We then evaluated in detail L-Tag specific cellular immune response in patients bearing BKV+ or BKV- PCa or BPH. The analysis of these data confirmed that a highly significant increase in IL-10 gene expression upon L-Tag peptide-pool stimulation was detectable in PBMC from patients bearing a BKV+ PCa as compared to those from patients bearing BKV- PCa ($p<0.0001$). In contrast, IFN-γ gene expression induced by L-Tag peptide-pool was modest, but detectable in PBMC from patients with BKV- PCa, and significantly higher than that detectable in similarly stimulated PBMC from patients bearing BKV+ PCa ($p=0.02$) (Figure 3a). On the other hand, expression of IFN-γ and IL-10 genes upon L-Tag peptide-pool stimulation was similar in PBMC from patients bearing BKV+ or BKV- BPH.

An analysis limited to patients with evidence of BKV L-Tag specific IgG also showed a highly significant ($p<0.0001$) predominance of IL-10 vs. IFN-γ gene expression in patients bearing BKV+ prostate lesions. In contrast, IFN-γ and IL-10 gene expression detectable upon L-Tag triggered PBMC stimulation were similar in L-Tag IgG+ patients with BKV+ BPH (Figure 3b).

Indeed, although IL-10 gene expression did not significantly differ between patients with BKV+ PCa or BKV+ BPH ($p>0.05$) (Figure 3a and 3b), most interestingly, IL-10/IFN-γ gene expression ratio was significantly ($p=0.04$) higher in BKV specific IgG+ patients bearing BKV+ PCa ($n=17$) than in BKV specific IgG+ patients with BKV+ BPH ($n=6$) (Figure 3c).

BKV+ PCa with BR evidence (n=7), as compared with those detectable in PBMC from patients bearing a BKV+ PCa with no BR evidence (n=8) (Figure 4a).

Importantly, in the former group, the extent of L-Tag specific IgG response was significantly correlated with both the IL-10/IFN-γ (ρ=0.8, p=0.02) and TGF-β1/IFN-γ (ρ=0.7, p=0.05) gene expression ratio induced in PBMC by L-Tag peptide stimulation (data not shown).

To verify these findings at the protein level, we cultured CD4+ T cells from patients bearing BKV+ PCa with (n=7) or without (n=8) evidence of BR in the presence of autologous DC pulsed with either HIV derived or L-Tag peptide pools and we analyzed IFN-γ, IL-10 and TGF-β1 release upon specific stimulation. In keeping with gene expression data (see above), we found that L-Tag specific stimulation in BR+ patients with BKV+ cancers induced a significantly ($p<0.01$) lower IFN-γ production and a significantly ($p=0.03$) higher TGF-β1 production, as compared to cells from BR- patients bearing BKV+ PCa (figure 4b). Although IL-10 release did not significantly differ in the two groups, IL-10/IFN-γ protein ratios were significantly ($p<0.01$) higher in BR+ patients bearing BKV+ PCa.

L-Tag peptide-pool stimulation expands $CD4^{+}CD25^{+(high)}FoxP3^{+}$ T cells in patients bearing BKV+ PCa with biochemical recurrence

The peculiar gene expression pattern induced by L-Tag in patients bearing BKV+ PCa, and, particularly, in BR+ patients, prompted us to investigate $CD4^{+}$ regulatory ($CD25^{+}FoxP3^{+}$) T cells in PBMC from these patients, both *ex vivo* and following L-Tag peptide-pool-specific stimulation. In particular, to adequately control our study, we focused on cells from L-Tag specific IgG+ patients bearing BKV+ PCa with or without BR (n=7 and n= 8, respectively) and from L-Tag specific IgG+ patients bearing BKV+ BPH (n=7) (Table 2).

Peripheral blood $CD4^+$ T cells from these patients were stained on their surface with anti CD25 mAb and intracellularly with anti Foxp3 mAb. No differences were detectable between patients with PCa, irrespective of BR, and patients with BPH regarding $CD4^+CD25^+FoxP3^+$ T cells *ex vivo* frequency (Figure 5a).

$CD4^+$ T cells from the different groups of patients under investigation were then cultured for 2-weeks in the presence of L-Tag pool, HIV pool or $HCMVpp65_{340\text{-}355}$ promiscuous peptide-pulsed autologous mDCs. $CD4^+CD25^{+(high)}$ populations in cultured cells were identified as shown in figure 5b. Following L-Tag peptide-pool stimulation, cultures from BR+ patients bearing BKV+ PCa contained significantly higher percentages of $CD4^+CD25^{+(high)}FoxP3^+$ cells, as compared to similarly stimulated cultures from BR- patients bearing BKV+ PCa ($p<0.001$) or to cultures from patients bearing BKV+ BPH ($p<0.001$) (Figure 5c). In contrast, $HCMVpp65_{340\text{-}355}$ promiscuous peptide failed to expand $CD4^+CD25^{+(high)}FoxP3^+$ cells to extents significantly higher than HIV pool peptides in cells from any of the patient population under investigation. Furthermore, $FoxP3^+$ protein expression upon L-Tag, but not $HCMVpp65_{340\text{-}355}$ or HIV pool peptide stimulation, was significantly enhanced, as evaluated by mean fluorescence intensity (MFI) measurement, in cells from BR+ patients bearing BKV+ PCa in comparison to cells from BR- patients bearing BKV+ PCa ($p<0.001$) or from patients bearing BKV+ BPH ($p=0.001$) (Figure 5d).

Functional effector memory phenotype (CD103) and regulatory activity of BKV L-Tag *in vitro* generated $CD4^+CD25^{+(high)+}CD127^{-(dim)}$ T cells

To investigate the potential functional relevance of the phenotypic modifications observed upon L-Tag stimulation, PBMC from BR+ patients bearing BKV+ PCa (n=3) were cultured in

the presence of L-Tag peptide-pool, HCMVpp65$_{340-355}$ promiscuous peptide and HIV peptide-pool, as detailed in "materials and methods". CD4^{+}CD25$^{+(high)}$ T cells were then sorted as CD127$^{-(dim)}$ and incubated in the presence of the antigens used for initial stimulation. High percentages of L-Tag and HCMVpp65$_{340-355}$ stimulated CD4^{+}CD25$^{+(high)}$CD127$^{-(dim)}$ T cells were able to produce IL-10, as detectable by intracellular staining (99.1±0.2% and 78.9±3.2%, respectively) (figure 6a). However, L-Tag stimulated CD4^{+}CD25$^{+(high)}$CD127$^{-(dim)}$ T cells showed a higher expression of CD103, a Treg effector memory marker (35), as compared to HCMVpp65$_{340-355}$ or HIV peptide-pool stimulated cells (Figure 6b). Most importantly, L-Tag expanded CD4^{+}CD25$^{+(high)}$CD127$^{-(dim)}$, IL-10-secreting T cells were able to inhibit the proliferation of autologous anti-CD3/CD28 stimulated CD4^{+}CD25^{-} T cells, in co-culture assays at a 0.5:1 ratio, to a significantly higher extent than HCMVpp65$_{340-355}$ expanded CD4^{+}CD25$^{+(high)}$CD127$^{-(dim)}$ T cells ($p<0.0001$) (Figure 6c and 6d).

Discussion

BKV infection has repeatedly been suggested to be associated with cancers of the genitourinary tract. However, possibly due to conflicting results regarding detection of specific sequences and proteins in human cancers, its oncogenic role is controversial (1).

In a recent past, we have analyzed in detail cellular immune responses to BKV L-Tag derived antigenic epitopes (39). Prompted by these studies we have now addressed key features of BKV L-Tag specific immune responsiveness in patients with PCa, and their association with clinical course.

Our data unravel that in patients with PCa, and, in particular, in those showing biochemical evidence of tumor recurrence (BR+), immune responsiveness to BKV L-Tag is differentially characterized by a number of conspicuous features, as compared with patients with no evidence of BR, or patients with BPH.

Indeed, in patients with BR+ PCa, a high titer of L-Tag specific IgG is significantly associated with a high IL-10/IFN-γ gene expression ratio as observed following *ex vivo* PBMC stimulation with L-Tag peptides. Furthermore, in these patients, L-Tag peptide stimulation of PBMC *in vitro* results in a significantly higher expansion of a $CD4^{+}CD25^{+(high)}FoxP3^{+}$ population as compared with patients bearing BKV+ PCa without BR evidence or with patients with BKV+ BPH. Notably, our data indicate that BKV L-Tag gene expression is detectable with similar frequency and at similar copy numbers in PCa and BPH tissues. Thus, we did not detect a preferential BKV gene expression in PCa (5), but rather a different pattern of L-Tag specific immune responses.

At difference with capsid proteins, polyomavirus L-Tag is not present in viral particles, but is only produced in infected cells and localizes in their nuclei. Therefore, while humoral response to

capsid proteins is widely detectable following infection, induction of L-Tag specific humoral responses is less likely to take place, unless cell death repeatedly occurs, which has been suggested to be the case in cancer tissues (38). Alternatively, we might hypothesize that only BKV abortive infections, e.g. those possibly leading to cancer transformation of infected cells, would permit L-Tag exposure to the immune system, thus favoring the induction of an antibody response (38). Indeed, our data show a significant association between L-Tag specific IgG levels and L-Tag molecular detection in patients with PCa, but not in those with BPH. In contrast, VP1 IgG levels were similar in patients with PCa and BPH, irrespective of BKV detection in prostate lesions, partially reflecting humoral responses observed against Merkel cell polyomavirus (MCV) VP1 in Merkel Cell Carcinoma (MCC), a cancer associated to MCV infection (50).

Cellular immune responsiveness to BKV L-Tag is also characterized by a number of peculiar features. In particular, in PBMC from patients with PCa, L-Tag peptide specific response is characterized by an IL-10 gene expression significantly higher than that of IFN-γ gene. In PBMC from patients with BPH a similar trend is detectable, whereas in cells from HD, IFN-γ gene expression is significantly higher than that of IL-10 gene. This gene expression pattern is unique to BKV L-Tag stimulated cells, because in PBMC from patients with PCa, BPH or from HD, other viral peptides (CEF pool) or PHA similarly induce an IFN-γ gene expression significantly higher than that of IL-10 gene. Therefore, the skewed responsiveness to BKV L-Tag cannot be merely attributed to a physiological decline of immune fitness, as frequently occurring in elderly individuals.

Taken together, these data suggest that the analysis of systemic BKV seroprevalence, as determined by IgG activity against viral capsid proteins, fails to support an association between BKV infection and PCa (38). Instead, humoral, and, most of all, cellular responsiveness to BKV

L-Tag skewed towards an immune regulatory gene expression profile, and characterized by a high IL-10/IFN-γ gene expression ratio, appear to be associated with PCa. Most conspicuously, this pattern of responsiveness is typically detectable in cells from patients with recurrent PCa.

Interestingly, recent data indicate that PBMC from patients with MCC might fail to produce IFN-γ in response to specific L-Tag peptides (28). In this case as well, infection by MCV was found to induce L-Tag specific humoral responses predominantly in cancer bearing patients. However, L-Tag stimulated IL-10 production was not explored.

It is tempting to speculate that the skewed cytokine gene expression signature typically detectable upon L-Tag peptide stimulation could be due to immune regulatory activities of cells preferentially secreting IL-10. However, while IFN-γ is only produced by T and NK cells, IL-10 can be produced by several cell types, including tumor cells (10, 21), virally infected cells (9), different types of activated antigen presenting cells and T cells.

We found similar numbers of circulating cells expressing classical $CD4^{+}CD25^{+(high)}FoxP3^{+}$ Treg phenotype in patients with PCa or BPH, irrespective of their BR and L-Tag molecular status. However, our data indicate that *in vitro* stimulation of PBMC from patients with recurrent BKV+ PCa, but not from patients with BKV+ PCa and no BR evidence or from patients with BKV+ BPH, results in the significant expansion of cells characterized by a typical Treg phenotype. These lymphocytes uniformly express IL-10, as detectable by intracellular staining, consistent with a predominant role in the elicitation of the skewed cytokine gene expression profile induced by BKV L-Tag in PBMC from patients bearing BKV+ PCa. Admittedly, the sole expression and/or release of IL-10 does not represent a reliable signature for regulatory T cell involvement (44) since it can be also induced in effector cells to reduce inflammation at the site of acute viral infections (49). However, the detection of this cytokine in combination with both

TGF-β1 and permanent FoxP3 expression is usually associated to regulatory functions (2, 52). Most importantly, L-Tag induced $CD4^+CD25^{+(high)}FoxP3^+CD103^+$ T cells also display a remarkable capacity to inhibit proliferation of autologous anti-CD3/CD28 stimulated $CD4^+CD25^-$ lymphocytes as compared to HCMVpp65 stimulated $CD4^+CD25^{+(high)}FoxP3^+CD103^{+/-}$ T cells, in accordance to recently published reports (46, 56). Collectively, these data hint to mechanisms potentially underlying our findings in *ex vivo* activated PBMC.

Immune regulatory activities driven by viral antigens and promoting the establishment of chronic infections have already been described (18, 20). These functions can be elicited by recruiting either $CD4^+$ or $CD8^+$ Treg, depending on type of infections (30). The involvement of both Treg subsets is dictated by HLA-restricted virus-specific stimulation taking place at the site of infection and presupposes both the expression of viral antigens and of Treg-derived cytokines, such as IL-10 and TGF-β1 (18, 30, 31). Moreover, circulating tumor associated antigen specific T cells with a regulatory phenotype ($CD25^{+(high)}FoxP3^+$) and the ability of secreting IL-10 and exerting suppressive functions have also been described in metastatic melanoma patients (51).

Still unclear is the molecular background preferentially favoring the expansion of these cells in patients bearing BKV+ PCa. It is of relevance, however, that prostate tissues appear to be characterized by features consistent with an immunosuppressive microenvironment. Infiltrating $CD4^+$ and $CD8^+$ T lymphocytes (TIL) are predominantly characterized by regulatory (32, 36) and functionally exhausted (PD-1+, B7-H1+) phenotypes (8, 17, 47). Furthermore, enhanced suppressive function of adaptive $CD4^+$ Treg has been observed in the peripheral blood of patients with PCa and found to correlate with metastatic behavior (55).

We previously showed that indoleamine-2, 3-deoxygenase (IDO) gene is frequently expressed to high extents in PCa (19). The specific gene product plays a key role in tryptophan

metabolism and its enhanced activities might result in both the depletion of an amino acid essential for lymphocyte metabolism and in the generation of toxic metabolites (53). In addition, arginase production by macrophages infiltrating prostatic tissues has been shown to favor the induction of anergy in resident lymphocytes (8). Therefore, in patients bearing a BKV+ PCa, presentation of L-Tag to T cells might occur in conditions likely favoring the generation of immune responses characterized by a high IL-10/IFN-γ gene expression ratio.

Most obviously, our data do not allow the postulation of any causal relationship between features of L-Tag specific immune responses, BKV infection and cancer. Nevertheless, they suggest the existence of a complex interaction of potential clinical relevance.

Our study has a major limitation. Indeed, although it capitalizes on the analysis of specific immune responses in a substantial number of patients, the number of informative cases, e.g. patients bearing BKV+ lesions with or without BR, is relatively modest. Therefore, additional investigations confirming these results in a larger number of cases are obviously warranted.

However, our data provide important novel contributions to the analysis of the increasingly puzzling relationship between BKV infection and PCa by identifying subpopulations of patients deserving major attention in translational research and, most importantly, by highlighting subtle peculiarities of immune responses against BKV L-Tag in patients with PCa, with previously unsuspected associations with the clinical course of this disease.

Acknowledgements

This work was partially supported by the Foundation for Research at the Medical Faculty, University of Zurich (Grant to GS) and the Swiss National Science Foundation (SNF) (grant 320000-122429/1 to MP and grant 320030-120320 to GCS).

We thank Dr. Gaetano Mauro for helping us in statistical data analysis and Mrs. Damina Balmer for her critical comments.

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Figure legends

Figure 1. BKV L-Tag DNA detection in tissue specimens and L-Tag or VP1 specific IgG response. (A) BKV L-Tag DNA was detectable in either PCa (n=18/43, 42%) or BPH (n=12/38, 32%) surgically excised lesions. Quantitative data, expressed as copy number/10^5 cells were comparatively analyzed in positive PCa and BPH tissues. To emphasize the specificity of BKV detection in prostate specimens, JCV L-Tag sequence was also investigated. Rare samples displayed JCV L-Tag sequences either in patients with PCa (n=1/40, 2%) or with BPH (n=1/36, 3%) with copy numbers markedly below 50 copies/10^5 cells, the arbitrary limit for BKV L-Tag DNA detection selected in our study (cut-off; dotted line). **(B)** BKV L-Tag and VP1 IgG titer in patients with either PCa or BPH, stratified according to BKV L-Tag DNA molecular testing in their tissues (positive lesions: BKV+ or +; negative lesions: BKV- or -). Cut-off (dotted line) was set at 0.04, the lowest OD_{492} sufficient to detect antibody activity. Boxes and whiskers (CI 95%) are also reported.

Figure 2. Cytokine gene expression upon *ex vivo* BKV L-Tag peptide-pool stimulation in PBMC from patients bearing PCa, BPH or healthy donors (HD).

(A) IFN-γ (white boxes) and IL-10 (grey boxes) cytokine gene expression was measured upon *ex vivo* BKV L-Tag peptide pool stimulation of PBMC from patients with PCa and PBH, stratified according to their L-Tag specific IgG activity (IgG+ ≥0.04). HIV peptide-pool stimulation was used as background to compute fold changes in specific gene expression (cytokine gene relative quantification ($2^{-\Delta\Delta Ct}$)). An arbitrary cut-off was set at 2-fold (dotted line). Boxes and whiskers (CI 95%) were reported to show the significance of differential gene expression for each cytokine based on patients' group stratifications. **(B)** Correlation (Spearman ρ) between cytokine gene

expression, as detected upon *ex vivo* L-Tag peptide-pool stimulation of PBMC from BKV L-Tag seropositive patients with PCa or BPH and L-Tag specific IgG. Ratios of IL-10/IFN-γ gene expression induced by L-Tag stimulation were plotted against L-Tag specific IgG activity for all IgG+ PCa (n=46; upper quadrant) and IgG+ BPH (n=38; lower quadrant) patients. Cut-off (dotted line) was set at 0.04 OD_{492} (see legend to figure 1). **(C)** Boxes and whiskers (CI 95%) for IFN-γ (white boxes) and IL-10 (grey boxes) cytokine gene expression, as observed in PBMC from L-Tag IgG+ patients with PCa (n=46), age-matched patients with BPH (n=38) and non age, gender-matched healthy donors (HD; n=8) following *ex vivo* stimulation with BKV L-Tag peptide pool, CMV/EBV/influenza virus peptide pool (CEF) or PHA. HIV peptide-pool stimulation was used as background to compute fold changes in specific gene expression (cytokine gene relative quantification ($2^{-\Delta\Delta Ct}$)). An arbitrary cut-off was set at 2-fold (dotted line).

Figure 3. Cytokine gene expression pattern upon *ex vivo* BKV L-Tag peptide-pool stimulation in L-Tag IgG+ patients bearing BKV+ PCa or BPH. (A) IFN-γ (white boxes) and IL-10 (grey boxes) cytokine gene expression was analyzed upon *ex vivo* BKV L-Tag peptide pool stimulation of PBMC from patients with PCa and PBH, stratified according to their BKV L-Tag DNA molecular testing (positive lesions: BKV+ or +; negative lesions: BKV- or -). HIV peptide-pool stimulation was used as background to compute fold changes in specific gene expression (cytokine gene relative quantification ($2^{-\Delta\Delta Ct}$)). An arbitrary cut-off was set at 2-fold (dotted line). Boxes and whiskers (CI 95%) were reported to show the significance of differences observed for each cytokine gene expression based on patients' group stratifications. **(B)** IFN-γ (white circles) and IL-10 (white squares) gene expression were measured upon L-Tag peptide-pool stimulation (cut-off 2-fold; dotted line) of PBMC from BKV+/IgG+ patients with PCa (n=17) or BPH (n=6).

(C) IL-10/IFN-γ gene expression ratio observed following L-Tag peptide stimulation of PBMC from BKV+/IgG+ patients bearing BKV+ PCa or BKV+ BPH. Boxes and whiskers (CI 95%) were reported to show differences of cytokine expression pattern between BKV+/IgG+ patients groups.

Figure 4. L-Tag peptide-pool induced cytokine gene expression in L-Tag IgG+ patients bearing BKV+ PCa with or without evidence of biochemical recurrence (BR)

(A) Ratios of IL-10/IFN-γ (grey boxes) and TGF-β1/IFN-γ (white boxes) gene expression, as detected following L-Tag peptide specific *ex vivo* stimulation of PBMC from L-Tag IgG+ patients bearing BKV+ PCa with (n=7) or without (n=8) BR evidence. HIV peptide-pool stimulation was used as background to compute gene expression fold changes ($2^{-\Delta\Delta Ct}$; cut-off 2-fold). Boxes and whiskers (CI 95%) were reported to show significant differences for both IL-10/IFN-γ and TGF-β1/IFN-γ gene expression ratios according to BR patients' stratifications. **(B)** IFN-γ (white bars), IL-10 (light grey bars) and TGF-β1 (dark grey bars) protein production from 2-week *in vitro* expanded PBMC from L-Tag IgG+ patients bearing BKV+ PCa with (n=7) or without (n=8) BR. HIV peptide-pool *in vitro* stimulation was used as control. Histograms ± standard errors are reported.

Figure 5. BKV L-Tag-specific expansion of $CD4^+$ T cell with a $CD25^{+(high)}FoxP3^+$ regulatory phenotype in L-Tag IgG+ patients bearing BR+BKV+ PCa

(A) Representative FACS density plot analysis of *ex vivo* FoxP3 expression in $CD4^+CD25^+$ T cells in PBMC from a PCa patient (upper panel) and a HD (lower panel). The histogram shows means ± standard errors of *ex vivo* detected frequencies of $CD4^+CD25^+FoxP3^+$ T cells in L-Tag

IgG+ patients with BR+/BKV+ PCa (dotted bar), BR-/BKV+ PCa (checkered bar) and BKV+ BPH (striped bar), as compared to HD (black bar). **(B)** Gating strategies for $CD4^+$ T cell with a $CD25^{+(high)}FoxP3^+$ phenotype, as detectable upon peptide stimulation. **(C)** Percentages of $CD4^+CD25^{+(high)}FoxP3^+$ T cells in PBMC from L-Tag IgG+ patients with BR+/BKV+ PCa (n=7), BR-/BKV+ PCa (n=8) or BKV+ BPH (n=7) upon HIV peptide-pool (circles) or L-Tag peptide-pool (squares) stimulation. $HCMVpp65_{340-355}$ promiscuous peptide (triangles). was also used as additional control on BR+/BKV+ PCa (n=6), BR-/BKV+ PCa (n=7) or BKV+ BPH (n=5) L-Tag IgG+ patients. **(D)** Mean Fluorescence Intensity (MFI) of FoxP3 intracellular staining of cultured cells from L-Tag IgG+ patients with BR+/BKV+ PCa, BR-/BKV+ PCa or BKV+ BPH, following HIV peptide-pool (circles), $HCMVpp65_{340-355}$ promiscuous peptide (triangles) or L-Tag peptide-pool (squares) *in vitro* stimulation. Overlaid histograms refer to FoxP3 MFI upon L-Tag peptide-pool and HIV peptide-pool (above) or L-Tag peptide-pool and $HCMVpp65_{340-355}$ promiscuous peptide (below) stimulations, as compared to isotype staining.

Figure 6. Generation of BKV L-Tag specific functional $CD4^+CD25^{+(high)}FoxP3^+$ T cells with effector-memory regulatory phenotype and suppressive activity.

(A) Intracellular IL-10 protein production in $CD4^+CD25^{+(high)}CD127^{-(dim)}$ T cells from one representative L-Tag IgG+ patient bearing a BKV+ PCa with BR, out of three studied, following L-Tag peptide-pool stimulation (right quadrant), as compared to $HCMVpp65_{340-355}$ promiscuous peptide stimulation (middle quadrant) and to negative control HIV peptide-pool stimulation (left quadrant). **(B)** Expression of the regulatory T cell activation marker CD103 in IL-10-secreting $CD4^+CD25^{+(high)}CD127^{-(dim)}$ T cells (see panel A) from one representative L-Tag IgG+ patient bearing a BKV+ PCa with BR, out of three tested. Overlaid histograms refer to L-Tag peptide-

pool stimulated cells (black line), HCMVpp65$_{340-355}$ stimulated cells (dark gray line) or negative control HIV peptide-pool stimulated cells (light grey line). **(C)** Proliferation index of anti-CD3/CD28 stimulated $CD4^{+}CD25^{-}$ T cells from one representative L-Tag IgG+ patient bearing a BKV+ PCa with BR, out of three tested, co-cultured with autologous L-Tag peptide-pool (circles) or HCMVpp65$_{340-355}$ induced IL-10-secreting $CD4^{+}CD25^{+(high)}CD127^{-(dim)}$ T cells (squares), or cultured alone (triangles) over 8 days. WST-1$^{(OD450)}$ values were plotted against days of incubation in co-cultures performed at a 0.5:1 ratio. **(D)** Mean±SE of proliferation index of anti-CD3/CD28 stimulated $CD4^{+}CD25^{-}$ T cells from the three IgG+ patients with BR+/BKV+ PCa tested, co-cultured as described above.

Table 1: Patients PSA, PCa Gleason score, L-Tag molecular testing and IgG serology

	PSA level	Gleason score	L-Tag DNA copies/10^5cells	L-Tag IgG (OD492)		PSA level	L-Tag DNA copies/10^5cells	L-Tag IgG (OD492)
PCa 1	7.50	7(3+4)	*nt*	+	BPH 1	12.75	-	+
PCa 2	5.22	9(5+4)	*nt*	+	BPH 2	*na*	-	+
PCa 3	4.60	7(4+3)	+	+	BPH 3	4.74	*nt*	+
PCa 4	5.30	7(4+3)	-	+	BPH 4	*na*	-	-
PCa 5	4.80	7(3+4)	-	+	BPH 5	*na*	-	+
PCa 6	10.70	7(3+4)	*nt*	+	**BPH 6**	2.96	+	+
PCa 7	7.64	7(4+3)	-	+	BPH 7	*na*	-	+
PCa 8	5.50	6(3+3)	+	+	BPH 8	18.60	-	+
PCa 9	4.10	7(3+4)	+	+	BPH 9	4.65	*nt*	+
PCa 10	7.50	8(3+5)	-	+	BPH 10	2.07	-	+
PCa 11	1.60	6(3+3)	*nt*	-	BPH 11	*na*	-	+
PCa 12	*na*	6(3+3)	*nt*	-	**BPH 12**	26.00	+	+
PCa 13	7.20	7(3+4)	-	-	BPH 13	1.79	+	-
PCa 14	7.80	7(3+4)	-	+	BPH 14	1.26	-	+
PCa 15	10.80	9(4+5)	*nt*	+	BPH 15	3.93	-	+
PCa 16	4.78	6(3+3)	+	-	BPH 16	*na*	-	+
PCa 17	*na*	*na*	*nt*	-	BPH 17	*na*	*nt*	+
PCa 18	4.17	9(4+5)	+	+	BPH 18	*na*	*nt*	+
PCa 19	25.60	7(4+3)	-	+	BPH 19	*na*	*nt*	+
PCa 20	4.70	7(4+3)	-	-	BPH 20	4.40	-	-
PCa 21	5.20	7(3+4)	-	-	**BPH 21**	17.00	+	+
PCa 22	*na*	7(3+4)	*nt*	+	BPH 22	1.16	-	+
PCa 23	4.05	7(3+4)	-	-	BPH 23	6.40	+	-
PCa 24	4.40	7(3+4)	-	-	BPH 24	1.36	+	-
PCa 25	35.00	7(4+3)	+	+	BPH 25	6.34	*nt*	+
PCa 26	61.30	6(3+3)	-	+	BPH 26	4.8	*nt*	+
PCa 27	*na*	*na*	+	+	**BPH 27**	10.10	+	+
PCa 28	9.20	7(3+4)	-	-	BPH 28	3.78	-	+
PCa 29	4.80	6(3+3)	-	-	BPH 29	4.26	-	-
PCa 30	*na*	7(4+3)	*nt*	+	**BPH 30**	1.66	+	+
PCa 31	6.59	7(3+4)	-	+	BPH 31	*na*	+	-
PCa 32	8.73	6(3+3)	*nt*	+	BPH 32	1.11	-	+
PCa 33	6.60	7(3+4)	+	+	BPH 33	*na*	-	+
PCa 34	3.44	7(3+4)	+	+	BPH 34	1.67	-	-
PCa 35	6.43	6(3+3)	-	+	BPH 35	*na*	*nt*	-
PCa 36	10.00	6(3+3)	*nt*	+	BPH 36	5.40	-	-
PCa 37	8.80	7(3+4)	+	+	BPH 37	2.12	-	+
PCa 38	7.46	*na*	*nt*	+	BPH 38	2.30	-	+
PCa 39	10.90	7(4+3)	+	+	**BPH 39**	8.14	+	+
PCa 40	106.00	9(4+5)	-	+	BPH 40	*na*	-	+
PCa 41	*na*	5(2+3)	-	-	BPH 41	*na*	*nt*	+
PCa 42	10.60	7(3+4)	*nt*	-	BPH 42	4.25	-	-
PCa 43	8.50	6(3+3)	-	+	BPH 43	1.24	-	+
PCa 44	7.20	7(4+3)	-	+	BPH 44	8.90	*nt*	+
PCa 45	11.52	6(3+3)	+	+	BPH 45	2.66	-	+
PCa 46	2.44	9(4+5)	+	+	BPH 46	2.30	+	-
PCa 47	*na*	*na*	*nt*	+	BPH 47	2.85	*nt*	+
PCa 48	48.00	7(4+3)	+	+	**BPH 48**	3.50	+	+
PCa 49	*na*	6(3+3)	+	+	BPH 49	0.36	*nt*	+
PCa 50	*na*	7(3+4)	-	+	BPH 50	15.70	-	+
PCa 51	*na*	*na*	*nt*	+				
PCa 52	4.70	7(3+4)	-	+				
PCa 53	2.56	6(3+3)	+	+				
PCa 54	6.78	7(3+4)	*nt*	+				
PCa 55	4.40	7(3+4)	-	+				
PCa 56	6.50	6(3+3)	-	-				
PCa 57	4.50	7(4+3)	+	+				
PCa 58	4.60	6(3+3)	+	+				
PCa 59	206.00	9(4+5)	-	+				
PCa 60	4.11	6(3+3)	*nt*	+				
	n=50	**n=55**	**n=43**	**n=60**		**n=36**	**n=38**	**n=50**
			BKV+ n=18	IgG≥0.04 n=46			BKV+ n=12	IgG≥0.04 n=38
			BKV - n=25	IgG<0.04 n=14			BKV - n=26	IgG<0.04 n=12

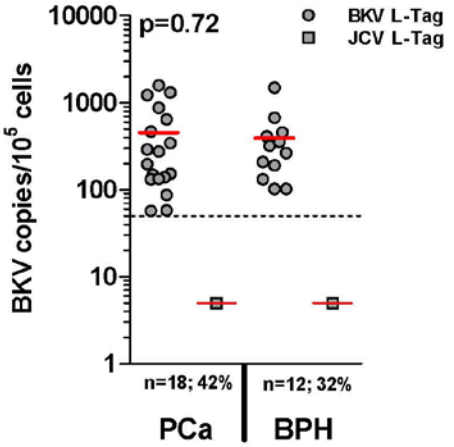
na: not available
nt: not tested
L-Tag BKV+ : evidence of L-Tag DNA detection in tissue specimens
L-Tag IgG+ : $OD_{492} \geq 0.04$
in bold: patients sharing L-Tag BKV+ specimens and IgG+ activity against L-Tag (IgG≥0.04): PCa n=17/18, BPH n=7/12; p=0.005 ($\chi 2$)

Table 2: Analysis of L-Tag IgG+ patients bearing BKV+ PCa with or without BR

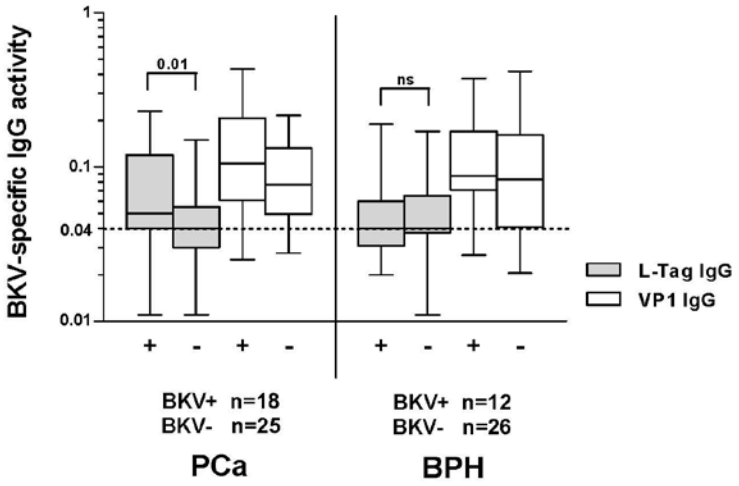
Patients	BR* (PSA ng/ml)	L-Tag DNA (copies/10^5cells)	L-Tag IgG (OD 492)	Patients	BR* (PSA ng/ml)	L-Tag DNA (copies/10^5cells)	L-Tag IgG (OD 492)
*IgG+/BKV+/BR+** PCa n=7*				*IgG+/BKV+/BR-*** PCa n=8*			
PCa 2	**6**	*nt*	+	PCa 9	-	+	+
PCa 8	**6**	+	+	PCa 16	-	+	-
PCa 18	**6**	+	+	PCa 25	-	+	+
PCa 27	**96**	+	+	PCa 33	-	+	+
PCa 37	**6**	+	+	PCa 34	-	+	+
PCa 48	**48**	+	+	PCa 39	-	+	+
PCa 49	**24**	+	+	PCa 46	-	+	+
PCa 57	**6**	+	+	PCa 53	-	+	+
PCa 60	**6**	*nt*	+	PCa 58	-	+	+
IgG+/BKV+ BPH n=7							
BPH 6	*na*	+	+				
BPH 12	*na*	+	+	*nt: not tested*			
BPH 21	*na*	+	+	*na: not available*			
BPH 27	*na*	+	+	**: biochemical recurrence (BR) upon 5-year follow up.*			
BPH 30	*na*	+	+	*** : BR+ (PSA≥0.2ng/ml)*			
BPH 39	*na*	+	+	**** : BR- (PSA<0.04ng/ml)*			
BPH 48	*na*	+	+	*in bold: timing of biochemical recurrence expressed in weeks*			

Figure 1

A


10000
1000
100
10
1
p=0.72
BKV L-Tag
JCV L-Tag
BKV copies/10^5 cells
n=18; 42%
n=12; 32%
PCa
BPH

B


1
0.1
0.04
0.01
0.01
ns
BKV-specific IgG activity
L-Tag IgG
VP1 IgG
+ - + -
+ - + -
BKV+ n=18
BKV- n=25
BKV+ n=12
BKV- n=26
PCa
BPH

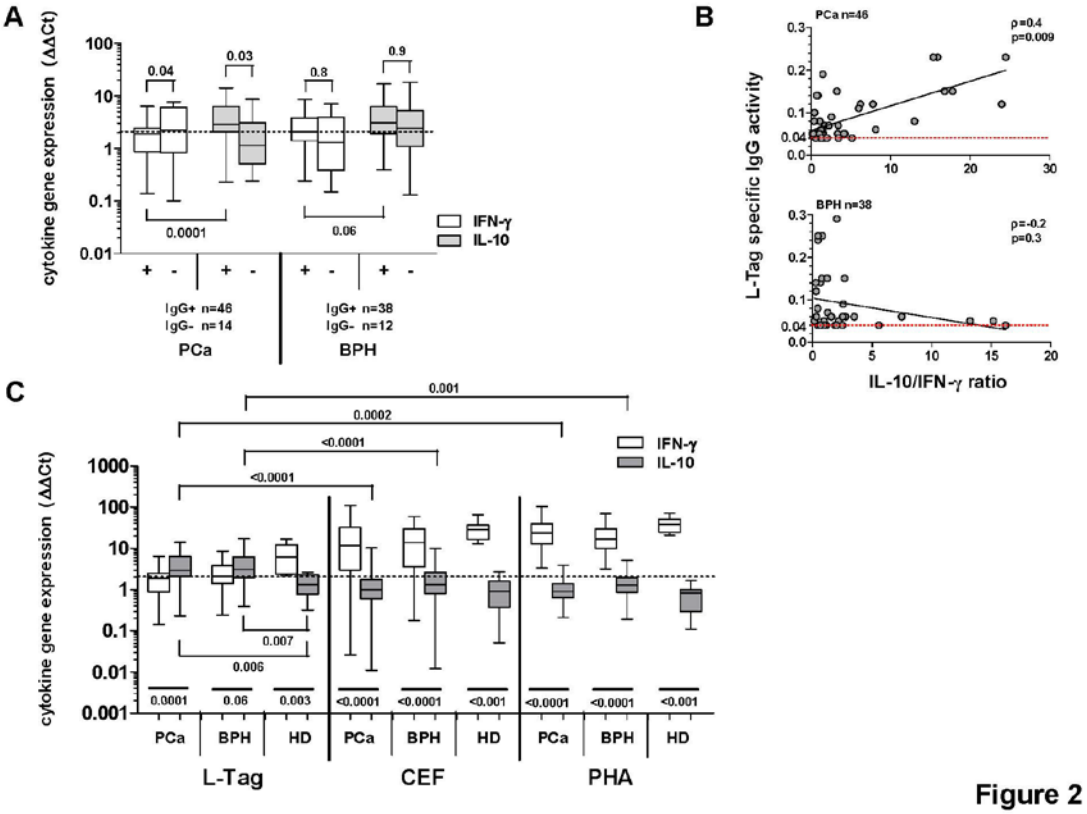

A
cytokine gene expression (ΔΔCt)
100
10
1
0.1
0.01
0.04
0.03
0.8
0.9
0.0001
0.06
IFN-γ
IL-10
+ - + - + - + -
IgG+ n=46
IgG- n=14
IgG+ n=38
IgG- n=12
PCa
BPH
B
PCa n=46
ρ=0.4
p=0.009
BPH n=38
ρ=-0.2
p=0.3
L-Tag specific IgG activity
IL-10/IFN-γ ratio
C
cytokine gene expression (ΔΔCt)
1000
100
10
1
0.1
0.01
0.001
0.001
0.0002
<0.0001
<0.0001
0.007
0.006
IFN-γ
IL-10
0.0001
0.06
0.003
<0.0001
<0.0001
<0.001
<0.0001
<0.0001
<0.001
PCa
BPH
HD
PCa
BPH
HD
PCa
BPH
HD
L-Tag
CEF
PHA

Figure 2

Figure 3

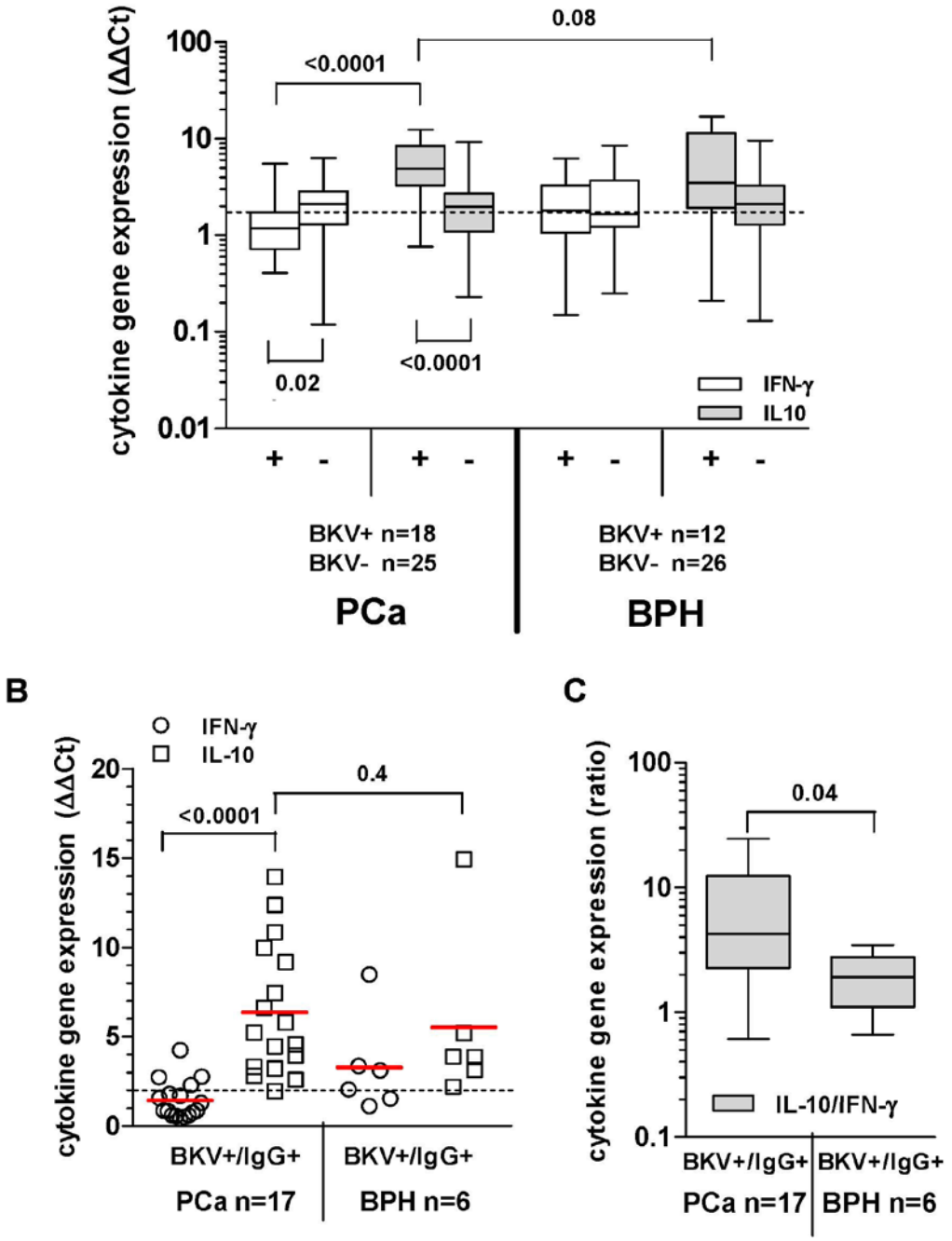
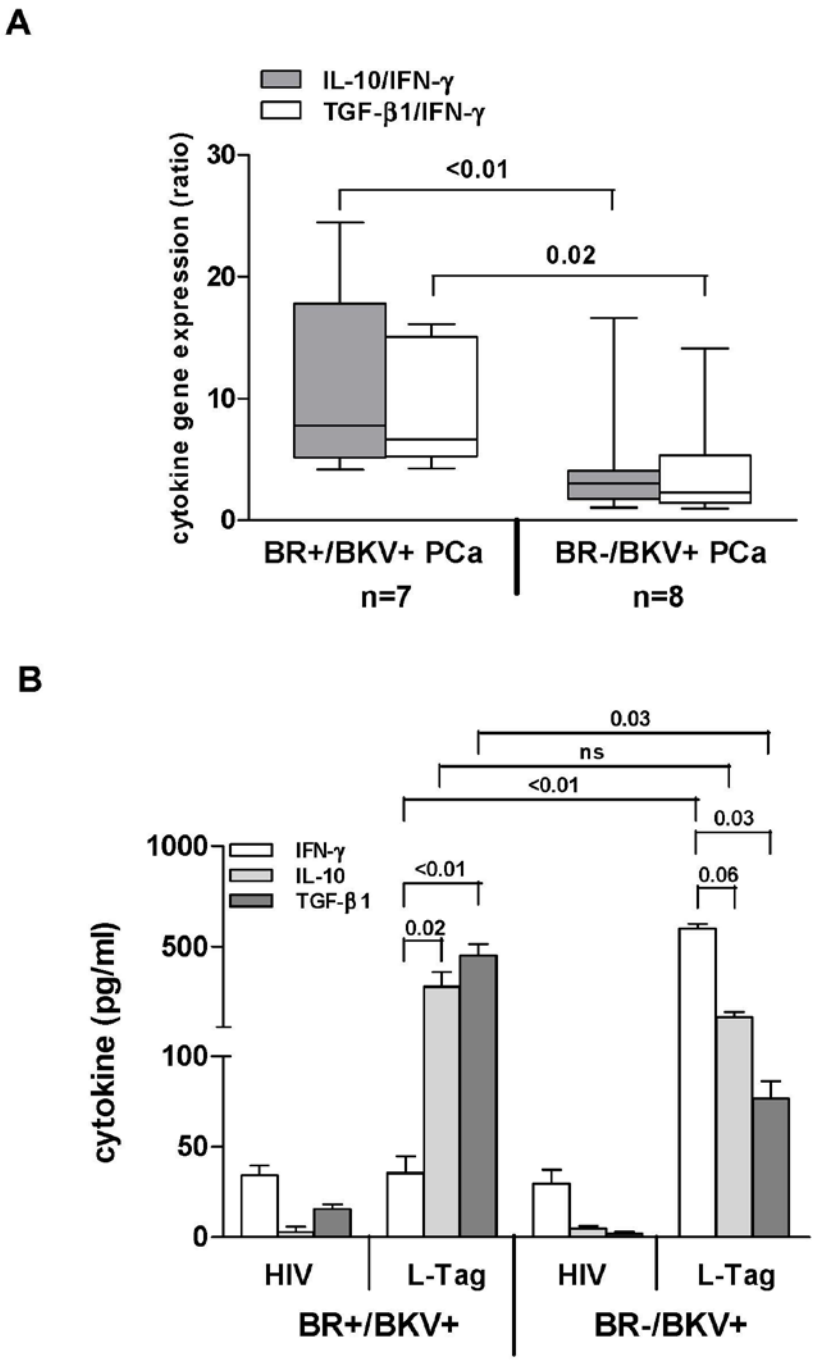

A
cytokine gene expression (ΔΔCt)
100
10
1
0.1
0.01
<0.0001
0.08
0.02
<0.0001
IFN-γ
IL10
+ - + - + - + -
BKV+ n=18
BKV- n=25
BKV+ n=12
BKV- n=26
PCa
BPH
B
IFN-γ
IL-10
cytokine gene expression (ΔΔCt)
20
15
10
5
0
<0.0001
0.4
BKV+/IgG+
BKV+/IgG+
PCa n=17
BPH n=6
C
cytokine gene expression (ratio)
100
10
1
0.1
0.04
IL-10/IFN-γ
BKV+/IgG+
BKV+/IgG+
PCa n=17
BPH n=6

Figure 4


A
IL-10/IFN-γ
TGF-β1/IFN-γ
cytokine gene expression (ratio)
30
20
10
0
<0.01
0.02
BR+/BKV+ PCa
n=7
BR-/BKV+ PCa
n=8
B
0.03
ns
<0.01
0.03
IFN-γ
IL-10
TGF-β1
<0.01
0.06
0.02
cytokine (pg/ml)
1000
500
100
50
0
HIV
L-Tag
HIV
L-Tag
BR+/BKV+
BR-/BKV+

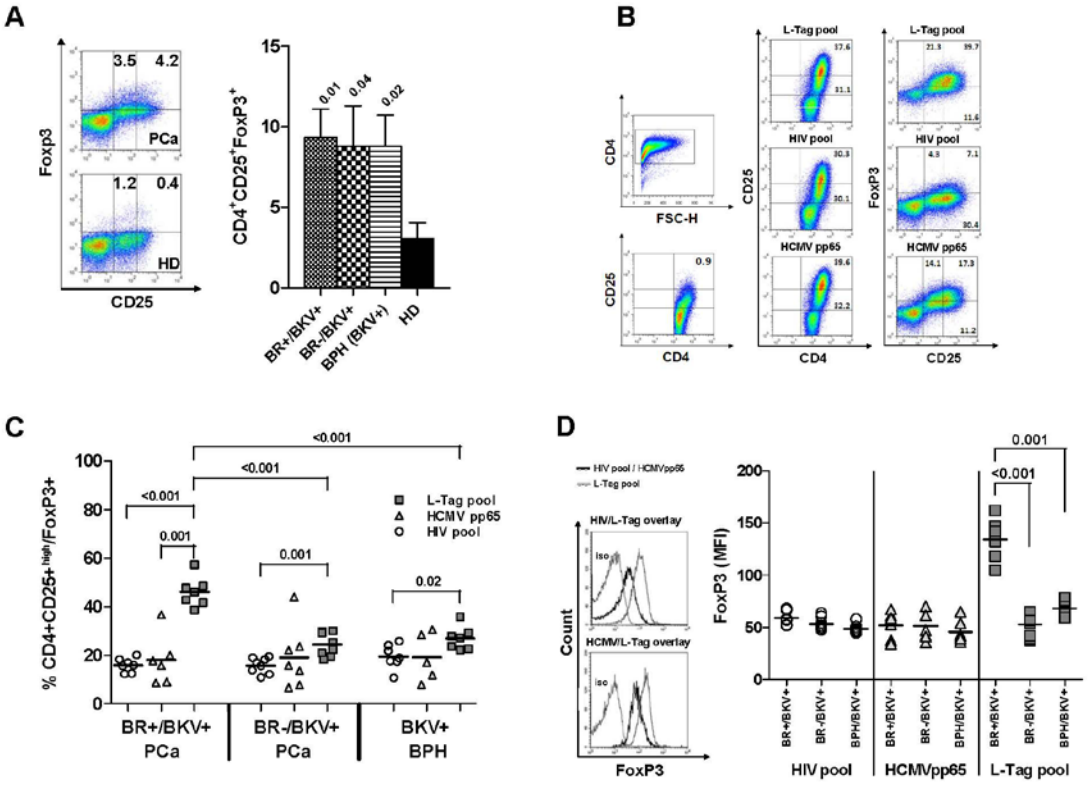
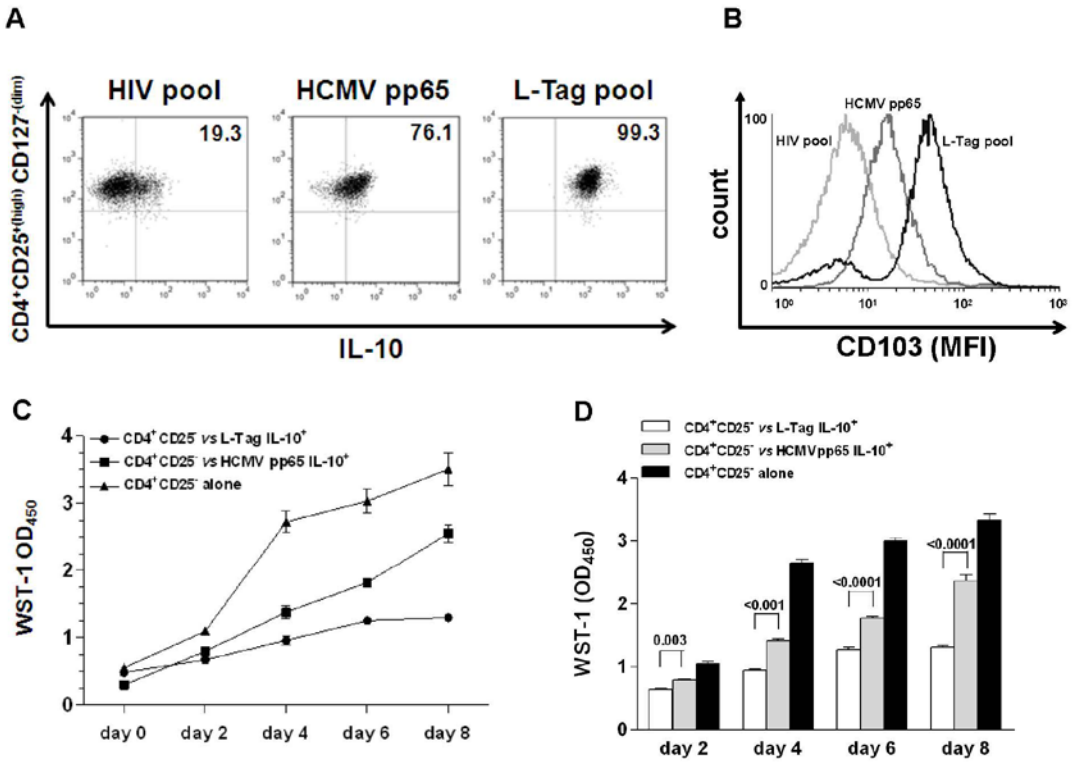

A
3.5
4.2
PCa
1.2
0.4
HD
Foxp3
CD25
CD4+CD25+FoxP3+
0.01
0.04
0.02
BR+/BKV+
BR-/BKV+
BPH (BKV+)
HD
B
L-Tag pool
HIV pool
HCMV pp65
CD4
FSC-H
CD25
FoxP3
C
% CD4+CD25+ high/FoxP3+
<0.001
0.001
0.02
L-Tag pool
HCMV pp65
HIV pool
BR+/BKV+ PCa
BR-/BKV+ PCa
BKV+ BPH
D
HIV pool / HCMVpp65
L-Tag pool
HIV/L-Tag overlay
HCMV/L-Tag overlay
Count
FoxP3
FoxP3 (MFI)
HIV pool
HCMVpp65
L-Tag pool

Figure 5


A
HIV pool
HCMV pp65
L-Tag pool
19.3
76.1
99.3
CD4+CD25+(high) CD127-(dim)
IL-10
B
HCMV pp65
HIV pool
L-Tag pool
100
0
count
CD103 (MFI)
C
CD4+ CD25- vs L-Tag IL-10+
CD4+ CD25- vs HCMV pp65 IL-10+
CD4+ CD25- alone
WST-1 OD450
day 0
day 2
day 4
day 6
day 8
D
CD4+CD25- vs L-Tag IL-10+
CD4+CD25- vs HCMVpp65 IL-10+
CD4+CD25- alone
WST-1 (OD450)
0.003
<0.001
<0.0001
<0.0001
day 2
day 4
day 6
day 8

3.4. Comprehensive characterization of BKV Large T antigen epitopes to promote the expansion of effectors T lymphocytes across a wide range of HLA class I and II antigens in prostate cancer

Giovanni Sais, Irina Banzola, Hans H. Hirsch, Tullio Sulser, Maurizio Provenzano

3.4.1. Materials and Methods

Donors and patients accrual and HLA genotyping

Twenty-nine consecutive patients diagnosed for either prostate cancer (PCa, n=19; Robot-assisted Laparoscopic Radical Prostatectomy) or benign prostate hyperplasia (BPH, n=10; Transurethral Resection of the prostate, TUR-P) were enrolled upon informed consent, as approved by the Ethical Committee of Zürich (KEK). All patients received post-surgical care at the Department of Urology, University Hospital of Zurich and relevant clinical data were collected by retrospective review of the patients' files. In addition, we collected blood (buffy-coats, Blutspende Zürich) from gender but not age-matched healthy donors (HDs, n=13).

A complete, high resolution, HLA genotyping (HLA-A*, HLA-B* and HLA-DRB1*) was performed by PCR with allele-specific sequencing primers (PCR SSP Protrans) according to the manufacturer's specifications.

Peptide selection, design and synthesis

Four major algorithms, IEDB (http://www.immuneepitope.org/home.do) (Vita, Zarebski et al. 2010), Bimas (http://www-bimas.cit.nih.gov/molbio/) (Parker, Bednarek et al. 1994), SYFPEITHI (http://www.syfpeithi.de) (Rammensee, Bachmann et al. 1999) and NetMHCII (http://www.cbs. dtu.dk/services/NetMHCII) (Nielsen, Lundegaard et al. 2008; Nielsen and Lund 2009) were employed to "in silico" predict the eleven HLA class II restricted BKV L-Tag candidate immunogenic peptides used in this study (human polyomavirus BK, strain Dunlop). Peptides were synthesized by GL Biochem Ltd. with purity ranging from 90% to 100%, as analyzed by high-performance liquid chromatography, dissolved in 100% Dimethyl Sulfoxide (DMSO) and stored at –70°C until use.

.PepMixes™ based on complete protein-spanning mixtures of overlapping peptides (lengths: 15aa, overlap: 11aa), such as BKV L-Tag peptides pool and HIV (B gag motif) peptides pool or PepMixes™ Cytomegalovirus/Epstein-Barr/Flu (CEF) peptides pool of 23 8-11mer peptides recognized by CD8 positive T cells and presented by 11 class I HLA-A and HLA-B alleles, were synthesized by JPT Peptide Technology (Berlin, Germany) and used as positive and negative controls. In addition, Phytohaemagglutinin (PHA) (Sigma Aldrich) and tetanus toxin universal T cell epitope (P2tt830-843) were used to test the integrity of our peptide screening.

Enzyme immunoassay (EIA)

BKV specific antibody detection in donor and patients sera was performed by enzyme immunoassay (EIA) with GST-BKV fusion protein, as reported in Leuenberger et al (Leuenberger, Andresen et al. 2007). Optical densities were measured using an automated plate reader (Tecan Group Ltd.) at 492 nm (OD_{492}). Affinity-purified GST was run as a negative control and subtracted from the GST-BKV L-Tag domain 1 (LTD1) signals. For every sample, the OD was determined by subtracting the corresponding GST background. The cut-off was defined as two standard deviations as above mean values of the negative controls. Therefore, all OD values <0.04 were considered negative. For confirmatory testing, we included three selected samples as references, (negative; <0.04, borderline positive; <0.1 and strongly positive; >0.1), indicating variation coefficients of <10%, which can cause minimal shifts in the groups.

Quantitative RT-PCR for BKV DNA detection

DNA from urine and serum of patients was extracted using the QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's instructions. The genome detection of polyomavirus BK was performed by qRT-PCR using a TaqMan assay targeting L-Tag. The assay, named T3a, consist of a primer/probe set which has been designed to reliably measure BKV L-Tag subtypes Ia, Ic, III, IV and VI (Hoffman, Cook et al. 2008). PCR amplification was set up according to standard real-time PCR protocols using a Corbett life science Rotor-gene 3000 instrument (Corbett Life science). Standard curves for the quantification of BKV L-Tag were generated using serial 10-fold dilutions of plasmid pBKV35-1 DNA (LGC Standards Sarl). For the purpose of this study, the limit of detection was arbitrarily set at 2 log copies/mL

T lymphocytes collection, separation and in vitro expansion

Peripheral blood mononuclear cells (PBMCs) isolated from venous blood of patients and donors by Ficoll-Hypaque density gradient centrifugation were resuspended in RPMI medium supplemented with 100µg/ml Kanamycin, 10mM Hepes, 1mM sodium pyruvate, 1mM Glutamax and non-essential amino acids (all from GIBCO Paisley), hereafter referred to as complete medium, supplemented with 5% human serum (Blutspende Zürich, Universitätsspital Zürich, Switzerland). For i*n vitro* culturing T cells were plated in complete

medium with 5% human serum in 24-well plates at a final concentration of $1x10^6$ cells/ml and were co-cultured (37°C, 5% CO_2 atmosphere) with autologous irradiated (750 sec in a gamma ray irradiator equipped with a Co radiation source emitting 100 rad/min) antigen presenting cells (APCs) previously pulsed for 4h with peptides (10µg/ml), either for priming (day 0) or for rounds of re-stimulation.

IFN-a dendritic cells were used as APCs (hereafter referred as IFNDCs). Briefly, CD14+ cells were purified from PBMCs by magnetic cell separation (Miltenyi Biotech) according to producers' protocols. Cells were then cultured at a final concentration of $1.5x10^6$ cells/well for 3 to 4 days in complete medium supplemented with 10% foetal calf serum, 0.004% mercaptoethanol, rhIFN-α (100000 UI/ml) and recombinant human granulocyte macrophage-colony stimulating factor (GM-CSF; 50 ng/ml) to generate mature IFNDCs.

Recombinant human (rh) IL-2 (Hoffmann-LaRoche) was added to the cultures at 1ng/ml, 1ng/ml and 5ng/ml, on days 3, 7 and 10, respectively. Cells were re-stimulated with specific peptide in the presence of irradiated APCs on day 7 of culture.

Antigenic stimulation and qRT-PCR analysis

Ex vivo induction of peptide-specific responses was attempted as follows. Briefly, 2×10^5 isolated PBMCs from patients and donors were incubated in 96 U-bottom well plates in 200 µl of complete medium supplemented with 5% human serum. After an overnight resting, each of the eleven L-Tag peptides selected by "in silico" prediction was in turn used to *ex vivo* stimulate (1 µM) the cells for 4 hours. As controls, BKV-L-Tag peptides pool, Phytohaemagglutinin (PHA), Tetanus toxin universal T cell epitope (P2tt830-843), and Cytomegalovirus/Epstein-Barr/Flu peptide-pool (CEF) were also used.

Total RNA extraction and cDNA synthesis were carried out following manufactures' instructions (MagMAX™-96 for Microarrays Total RNA Isolation Kit, AMBION and High Capacity cDNA Reverse Transcription Kit, Applied Biosystem). Quantitative real-time PCR (qRT-PCR) assays were performed as previously described on a Rotor-gene 3000 detection system (Corbett Life Science) using TaqMan® gene expression PCR Master Mix Reagents Kit and sets of primers and probes (Applied Biosystems) from cytokine genes (IFN-γ, TNF-α, IL-2, IL-10, TGF-β1), lytic machinery components (Granzyme, Perforin) and factors regulating cytolysis/apoptosis (Fas-L and PD-1).

The $2^{-\Delta\Delta Ct}$ method [$\Delta\Delta C_T = (C_T,$ cytokine $- C_T,\beta\text{-actin})_{induction} - (C_T,$ cytokine $- C_T,\beta\text{-actin})_{baseline},$ where C_T is the mean cycle times of the triplicate well readings] was employed to

compute fold changes of cytokine gene expression. The HIV peptide-pool with B gag motif was used as negative control to provide the baseline allowing the $\Delta\Delta C_T$ calculation. An endogenous reference gene (β-actin) was used to normalize target gene expression. Two-fold increase was the threshold for significant fold changes.

Cytokine intracellular staining and T-cell phenotype

Peptide expanded PBL ($1x10^6$) were rested overnight and then stimulated with 5 $x10^4$ peptide-pulsed (10 µg/ml) IFNDCs. Two hours after induction, 1µg/ml of Brefeldin A (Sigma Aldrich) was added to the culture. For CD107 detection, anti-CD107a antibody (BD Biosciences) was added at the beginning of the stimulation in the presence of GolgiStop (BD Biosciences) (1µg/ml). After four additional hours, cell incubation was stopped by washing cells with cold phosphate-buffered saline (PBS). Pellets were re-suspended in 1 ml PBS containing 1 mM EDTA and 0.5% FCS and cells were stained extracellularly with 10 µl of fluorescent mAbs against CD4+, CD8+, CD25, CD45RA, CD62L, CD69 (BD Bioscience) for 15 min at 4°C in the dark. Cell fixation was performed with 2 ml of BD FACS Lysis Solution (BD Bioscience), according to the producers' instructions. Cellular permeabilization was performed by re-suspending cells in 500 µl of FACS Permeabilizing Solution 2 (BD Bioscience) at 1:10 dilution in Diethyl Pyrocarbonate (DEPC) water, at room temperature for 10 min. For intracellular staining, either 5µl of human anti-IFN-γ or 5µl of human anti-IL-2 mAbs were used as test and mouse IgG1 isotype-(BD Bioscience) was used as control. Cells were then incubated for 30 min at 4°C in the dark. Samples were acquired on a FACSCalibur flow-cytometer (BD Bioscience) and analyzed by Flowjo software (Tree Star).

Monoclonal T-Cell Sorting Using an IFN-γ Secretion Assay

Two-week L-Tag peptides *in vitro* cultured CD8+ and CD4+ T cells were re-challenged with autologous APCs pulsed with relevant peptides or control pool at a ratio of 1:2 for a maximum of 5 h. Cell surface detection of IFN-γ-secreted molecules was performed according to the manufacturer's instructions (Miltenyi Biotech) as follows. Briefly, cells were labeled for 5 min at 4°C with an IFN-γ-specific high-affinity capture matrix (bispecific Ab-Ab conjugate directed against CD45 and IFN-γ). Cells were then transferred into a 37°C warm medium for 45 min to permit secretion of IFN-γ, washed and stained for 30 min at 4°C with PE-labeled anti-IFN-γ mAb (Miltenyi Biotech). Subsequently, cells were washed and

magnetically labeled for 15 min at 4°C with anti-PE Ab microbeads (Miltenyi Biotec) and PE-labeled IFN-γ secreting cells were enriched through two rounds of positive selection by magnetic cell sorting (Miltenyi Biotech) to generate peptide-specific oligoclones to use for functional assays. Part of each sample was analyzed by flow cytometry to assess the viability of CD4+ and CD8+ IFN-γ secreting T cells.

LDH release cytotoxic assay

The cytotoxic activity of IFN-γ secreting T-cell oligoclones was measured using lactate dehydrogenase (LDH) release assays (Promega). As target cells, peptide- or control pool-pulsed autologous IFNDCs were cultured withCD4+ or CD8+ T cell suspensions at 50:1, 20:1, 10:1, 5:1 and 1:1 effector:target (E:T) ratio, in triplicate and incubated at 37°C, 5% CO_2 atmosphere for 6h. Thereafter, 100 µl/well supernatant was carefully removed and transferred into corresponding wells of an optically clear 96-well flat bottom microplate. 100 µl reaction mixtures was added to each well and incubated for 25 min at room temperature in the dark. Absorbance at 490 was measured using a microplate enzyme-linked immunosorbent assay reader. The percentage of specific lysis was calculated as follows: (experimental release - spontaneous release)/ (maximum release - spontaneous release)*100. Maximum release was obtained by adding 100 µL of lysis solution to the 100 µL medium containing target cells. Spontaneous release was consistently less than 15% of maximum release in all assays.

Statistical analysis

All data were analyzed using SPSS data editor 19 and Graph Pad Prism 5.0 statistical software. Data were reported as mean ± S.D. or median and ranges where appropriate. Differences between two groups were analyzed using Student's t-tests. Differences between more than two groups were analyzed using repeated measures ANOVA and Bonferroni's multiple comparisons as a post hoc test. Two sided p-values of < 0.05 (CI 95%) were considered statistically significant.

3.4.2. Results

3.4.2.1. Peptide sequences characterization and algorithm predictions

To identify candidate immunodominant peptides within Large T antigen (Table III - 1) we used an *in silico* prediction methodology that takes into account peptide processing (immuno-proteasome cleavage and TAP transport) and HLA-binding affinity. We thus employed different algorithms, such as the Epitope Database and Analysis Resource (IEDB), SYFPEITHI, Bimas and NetMHCIIpan Database.

HLA class I binding affinity was then analyzed by combining IEDB, SYFPEITHI and Bimas datasets, giving priority to peptides with higher affinity. We thus selected nonamer (9mer) peptides with the highest probability to bind HLA class I molecules within at least 34 subtypes (15 HLA-A and 19 HLA-B). The predicted IEDB output is given in units of IC_{50}nM. Therefore, peptides with IC_{50} values <50 nM bear high affinity, <500 nM intermediate affinity and <5000 nM low affinity properties, respectively. Bimas scores nonamer (9mer) and decamer (10mer) peptides according to their half-time disassociation from the HLA binding groove, while SYFPEITHI considers the amino acid positions as anchor residues for peptide binding to its corresponding HLA molecules.

Based on score datasets, we were able to select several HLA-A and B restricted 9mer peptides predicted to be immunogenic. Interestingly, the majority of these 9mer peptides were amino acid sequences nested, in number of 3 or higher, in same regions of the L-Tag, thus characterizing, by tiling at 2 to 4 amino-acid pace, putative HLA class II restricted peptides (Table II-1). Therefore, eleven 15-18mer peptides (BK1 L-Tag_{27-41}, BK2 L-Tag_{69-85}, BK3 L-$Tag_{148-164;}$ BK4 L-$Tag_{172-185}$, BK5 L-$Tag_{196-209}$, BK6 L-$Tag_{212-226}$, BK7 L-$Tag_{398-414}$, BK8 L-$Tag_{410-426}$, BK9 L-$Tag_{521-537;}$ BK10 L-$Tag_{531-545}$, BK11 L-$Tag_{570-587}$) were those nesting the highest numbers of HLA class I restricted potentially immunogenic 9mer peptides.

We then considered these regions for HLA class II binding affinity by employing NetMHCIIpan and IEBD databases. Prediction was run on a total of 13 HLA class II loci. In brief, in the IEDB database, four methods (ARB, combinatorial library, SMM_align and Sturniolo) provide peptide scores and percentile rank of each 9mer core sequence identified in larger putative peptides (usually 15mer sequences selected by the algorithm) in relation to the HLA allele the prediction was made. Prediction is generated by comparing the peptide's score against the scores of five million random 15mer peptides selected from SWISSPROT database. The median percentile rank of the four methods is then used to generate the rank for consensus score. A small numbered percentile rank indicates high affinity. NetMHCIIpan method is used to calculate consensus score when other methods, such as SMM_align,

and/or Sturniolo are not available for a particular HLA allele. Differently, NetMHCIIpan is used as second or third choice.

3.4.2.2. HLA typing, BKV L-Tag serology and molecular testing in donors and patients

A complete high resolution HLA class I and II genotyping was performed by sequence-specific PCR using genomic DNA. HLA specificities for class I alleles, loci A and B, and for class II alleles, locus DRB were considered (Table II-2).

Humoral response against BKV L-Tag was tested in blood serum of both patients and donors. BKV L-Tag IgG seroprevalence was observed in 16/19 (84.21%) patients with PCa, in 9/10 (90%) patients with BPH and 10/13 (76.92%) HDs. We also tested BK viral load in urine (viruria) and in sera (viremia) of same individuals by using real-time polymerase chain reaction targeting L-Tag. Asymptomatic BK viruria was observed in one patient (PCa-196: 854 copies/ml), whereas it was absent in all BPH patients and donors with available urine (n=4). Similarly, BK viremia was detected in one patient (PCa-9: 218 copies/ml) (Table II – 2).

3.4.2.3. Systemic cellular immune response upon BKV L-Tag peptide-pool induction

In order to define the systemic cellular immune response against BKV L-Tag in both groups of patients, as compared to gender- but not age-matched HDs, we tested cytokine gene expression upon *ex vivo* antigenic stimulation by using the BKV L-Tag PepMix (a complete protein-spanning mixture of 170 15mer overlapping peptides at 11 amino-acid pace). Freshly isolated PBMCs of 16 patients with PCa, 10 patients with BPH and 10 HDs were 4-hour *ex vivo* stimulated with L-Tag peptide-pool, Phytohaemagglutinine (PHA), and Cytomegalovirus/Epstein-Barr/Flu peptide-pool (CEF). The HIV peptide-pool with B gag motif was used as negative control to provide a baseline allowing the calculation of cytokine gene expression fold changes. A panel of gene encoding pro-inflammatory (IFN-γ, TNF-α) and regulatory (IL-10, TGF-β1) cytokine was used as readout.

T cell immune reactivation is generally observed in individuals carrying positive antibody activity against viral antigens they have experienced when portions of those antigens (peptides) are used to both *ex vivo* and *in vitro* recall specific cellular immune responses. Thus, testing patients and donors accrued, cytokines gene expression upon L-

Tag peptide-pool induction exceeded the arbitrary threshold level of 2-fold in all the BKV L-Tag seropositive subjects, as compared to BKV L-Tag seronegative counterparts (Figure II-2), thus confirming the integrity of our experimental approach that identify specific antigenic reactivation in seropositive subjects upon short-time *ex vivo* inductions.

According to peculiar differences observed between proinflammatory and regulatory cytokine gene expression upon L-Tag peptide-pool stimulation, in particular IFN-γ (PCa patients: mean±SD: 1.33±0.97, median: 1; BPH patients: mean±SD: 1.8±1.3, median: 2) and IL-10 (PCa patients: mean±SD: 4.4±3.5, median: 2.1; BPH patients: mean±SD: 2.5±3.4, median: 1) (Figure II-3), a systemic differential pattern of immune responsiveness seems to be fostered by L-Tag in seropositive PCa patients (n=13, p=0.004) but not in seropositive BPH patients (n=9, p=0.59), despite a pro-inflammatory impairment, considering both IFN-γ and TNF-α gene expression, was seen in both groups of patients. As expected, a pro-inflammatory cytokines gene expression significantly exceeded a regulatory cytokine gene expression (IFN-γ: mean±SD: 13.2±15.3, median: 6.7; IL-10: mean±SD: 1.6±10.7, median: 1.8; p=0.048) in L-Tag peptide-pool induced seropositive HDs (n=8) (Figure II-4). In addition, the relevant pro-inflammatory cytokine gene expression seen in PCa patients after stimulation with control peptide-pool CEF (IFN-γ: mean±SD: 14.30±19.6, median: 5.54) better stress that the cytokine skew due to L-Tag cannot be merely ascribed to physiological decline of immune fitness frequently occurring in elderly individuals (Figure II-3).

An additional analysis of the regulatory cytokine TGF-β1 expression upon L-Tag peptide-pool stimulation did not corroborate IL-10 gene expression data, although some samples were positive for TGF-β1 gene expression in both PCa patients (mean±SD: 2.3±2.14, median: 2) and BPH patients (mean±SD: 1.97±2.2, median: 1.62) (p=0.74) (Figure II-3).

3.4.2.4. Ex vivo immune responsiveness to confirm the potential immunogenicity of in silico predicted L-Tag peptides

To determine whether portions of L-Tag, such as single peptides, could confirm or revert the skewed immune responsiveness observed in patients upon L-Tag peptide-pool induction, the eleven *in silico* selected peptides were employed to characterize T cells responses upon their stimulation on freshly isolated PBMCs from same accrued patients and donors (see 3.4.2.3). The tetanus toxin universal T cell epitope ($P2tt_{830-843}$) was included among the positive controls used in this study. Transcript productions of relevant pro-inflammatory (IFN-γ, TNF-α) and regulatory (IL-10, TGF-β1) cytokine were thus compared among groups of patients and healthy donors.

Four peptides out of the eleven predicted to be immunogenic (BK1 L-Tag$_{27-41}$; BK4 L-Tag$_{172-185}$; BK6 L-Tag$_{212-226}$; BK10 L-Tag$_{531-545}$) induced a significant pro-inflammatory immune response (as detected by IFN-γ and TNF-α gene expression) in about half (49.2%) of BKV seropositive PCa patients (BK1 L-Tag$_{27-41}$: 45%, mean 4 ± 5.7, median 1.8 folds; BK4 L-Tag$_{172-185}$: 54%, mean 2.2 ±1.5, median 2 folds; BK6 L-Tag$_{212-226}$: 54%, mean 4.4 ± 6.7, median 2.3 folds; BK10 L-Tag$_{531-545}$: 44%, mean 3.4 ± 3.8, median 1.8 folds) (Figure II – 5) and in almost 70% of gender-matched HDs (mean 66.75%; range 55-78%). Of relevance, no significant concomitant induction of regulatory cytokines (IL-10 and TGF-β1) was detected (PCa patients: BK1 L-Tag$_{27-41}$: 23%, mean 1.5 ± 1, median 1 folds; BK4 L-Tag$_{172-185}$: 20%, mean 1.4 ± 0.8, median 1 folds; BK6 L-Tag$_{212-226}$: 12%, mean 1.8 ± 4.4, median 1 folds; BK10 L-Tag$_{531-545}$: 10%, mean 1.9 ± 3.7, median 1 folds) (Figure II – 6). Similar data, although at lesser non significant extent, were obtained from BPH patients (pro-inflammatory gene expression (pge): mean 40%; range 35-45%; regulatory gene expression (rge): mean 20%; range 15-25%; data not shown).

Two peptides, BK7 L-Tag$_{398-414}$ (pge: 27%, mean 1.93 ± 1.87, median 1.13 folds; rge: 61%, mean 3.01 ± 2.66, median 2.2 folds) and BK11 L-Tag$_{570-587}$ (pge: 31%, mean 1.67 ± 1.47, median 1.1 folds; rge: 54%, mean 4.69 ± 7.56, median 2.1 folds) confirmed the cytokine skew observed in PCa patients upon L-Tag peptide pool induction (Figure II – 5/6). Notably, two 9mer peptides, BK L-Tag$_{406-414}$ and BK L-Tag$_{579-587}$, nested within BK7 L-Tag$_{398-414}$ and BK11 L-Tag$_{570-587}$, respectively, have been previously see by us to trigger an immune-regulatory T cell response in PCa patients (Sais et al: data not shown), whereas they were documented to induce pro-inflammatory responses in BKV seropositive healthy donors (Provenzano, Bracci et al. 2006).

In addition to BK1 L-Tag$_{27-41}$, BK4 L-Tag$_{172-185}$, BK6 L-Tag$_{212-226}$ and BK10 L-Tag$_{531-545}$, three more peptides, BK3 L-Tag$_{148-164}$, BK8 L-Tag$_{410-426}$ and BK9 L-Tag$_{521-537}$, were able to specifically recall immune response, but only in HDs.

3.4.2.5. Phenotypic characterization of IFN-γ- and IL-2-producing BKV pL-Tag expanded T cells

Cytokine gene expression data indicated that BK1 L-Tag$_{27-41}$, BK4 L-Tag$_{172-185}$, BK6 L-Tag$_{212-226}$ and BK10 L-Tag$_{531-545}$ peptides were able to efficiently *ex vivo* recall immune activity in both seropositive patients and healthy donors. To gain insight into functional features of peptide-specific T cells reactive against BKV L-Tag, both freshly isolated CD4+ and CD8+ T cells from same patients with PCa and healthy donors were 2-week peptide *in*

vitro expanded. Patients bearing BPH were excluded due the weak cytokine gene expression detected in all individuals reacting to each of the four peptides, as compared to HDs. Cytokines production was then analyzed according to cell phenotype. Stimulation was performed with a mini-pool consisting of BK1 L-Tag$_{27-41}$, BK4 L-Tag$_{172-185}$, BK6 L-Tag$_{212-226}$ and BK10 L-Tag$_{531-545}$ (hereafter referred as pL-Tag). We considered as positive response any pL-Tag-induced cytokine production (IFN-γ and IL-2) exceeding 3-fold the background (2-week *in vitro* expansion with pL-Tag and re-challenging with HIV peptide-pool).

Eight subjects (5 PCa patients and 3 HDs) showed pL-Tag-specific CD4+ T cells, as defined by IFN-γ production, and among them five (4 PCa patients and 1 HD) showed pL-Tag specific CD8+ T cells. Percentage of IFN-γ-producing CD4+ T cells ranged from 0.8 to 8.6% (mean 3.1±2.5%, median 2.6%) (Figure II – 7A), while percentage of IFN-γ-producing CD8+ was less relevant and ranged from 0.54 to 1.44% (mean 1.1±0.4%, median 1.2%) (Figure II – 7B).

CD62L and CD45RA are markers in use to characterize CD4+ and CD8+ T cells phenotype. Indeed, four distinct T-cell subsets can be described: CD45RA+ CD62L+^{high} (naive), CD45RA– CD62L+^{high} (T_{CM}: central memory), CD45RA– CD62L+^{low} /CD62L- (T_{EM}: effector/memory) and CD45RA+ CD62L+^{low} /CD62L- (T_{EMRA}: effector/memory) (Seder and Ahmed 2003) (Lefrancois and Marzo 2006) (Sallusto, Geginat et al. 2004). In our analysis, IFN-γ-producing CD4+ and CD8+ from either PCa patients or HDs were found to belong to the effector memory T cells subpopulation. Among them, IFN-γ-producing CD8+ T cells from two PCa patients showed a clear CD45RA+ T_{EMRA} phenotype (Figure II – 8, 9).

To determine the effector function of pL-Tag-specific IFN-γ producing CD4+ and CD8+ T cells, CD107a expression was used to evaluate their cytolytic potential. CD107a is a lysosomal-associated membrane protein that has been described as a marker of CD8+ T-cell degranulation following stimulation. It is expressed on the cell surface after lysosomal membrane fusion and it is a necessary precursor of cytolysis (Betts, Brenchley et al. 2003). In this specific setting, both pL-Tag-expanded IFN-γ-producing CD8+ and CD4+ T cells showed a significant increase in surface mobilization of CD107a, as compared to IFN-γ negative peptide-stimulated counterparts (Figure II – 8, 9) and in accordance to significant degranulation following mitogenic (PHA) stimulation (Figure II-10).

Given the decisive role of helper T cells in the optimal expansion of CTLs, we also assayed the ability of pL-Tag to induce and expand IL-2-producing CD4+ T cells. As expected, all batches of cells from either PCa patients or HDs that were responsive to pL-Tag-induction (n=8) were IL-2-producing CD4+ T cells with the peculiar helper phenotype (CD25+ CD69+) (Figure II – 8).

3.4.2.6. Functional features and peptide-specific HLA restrictions of CTL polyclones generated from single IFN-γ secreting T cells

In order to identify cognate recognition of each of the four peptides contained in the pL-Tag (BK1 L-Tag$_{27-41}$, BK4 L-Tag$_{172-185}$, BK6 L-Tag$_{212-226}$ and BK10 L-Tag$_{531-545}$), pL-Tag *in vitro* induced T lymphocytes were re-challenged with single peptides and both killing phenotype and killing activity were performed. Thus, two-week *in vitro* expanded CD4+ and CD8+ T cells with a significant mobilization of CD107a upon pL-Tag induction (CD4+/pL-Tag/Pca-196, CD4+/pL-Tag/PCa-223, CD4+/pL-Tag/HD-GS and CD8+/pL-Tag/PCa-196) showed the following peptide-specific T cells responses: CD4+/BK1 L-Tag$_{27-41}$/Pca-196, CD4+/BK10 L-Tag$_{531-545}$/PCa-223, CD4+/BK6 L-Tag$_{212-226}$/HD-GS and CD8+/BK4 L-Tag$_{172-185}$/Pca-196 (Figure II - 11). Therefore, we generated CTL polyclones sorting IFN-γ secreting T-cells from each batch of T cells responsive to single peptide stimulation, as reported above, and evaluated their capability of exerting cytotoxic activity on peptide-pulsed autologous IFNDCs. Three CTL polyclones generated by IFN-γ-secreting T cells (CD4+/BK1 L-Tag$_{27-41}$/Pca-196, CD4+/BK6 L-Tag$_{212-226}$/HD-GS, and CD8+/BK4 L-Tag$_{172-185}$/Pca-196) exerted cytotoxic activity with a delta specific lysis (ΔSL) >40% at 10:1 ratio, whilst CD4+/BK10 L-Tag$_{531-545}$/PCa-223 at 10:1 ratio did not reach 20% (Figure II – 12A).

Cells were in parallel analyzed for the expression of genes encoding molecules mediating cytotoxicity. Three CTLs mediated cytotoxic mechanism were considered: exocytosis of lytic proteins (perforin, granzymes), receptor-ligand binding (Fas-L) and TNF-α mediated cytolysis/apoptosis. As shown in Figure II -12B, there was a significant expression of granula-dependent factors (perforin and granzyme) and receptor-mediated cytotoxic effectors molecules in all CTL polyclones tested. In addition, to settle CTLs exhaustion, we tested the expression of programmed death 1 receptor (PD-1). We could detect significant expression of PD-1 in none of the CTL polyclones analyzed, thus confirming their potential to retain functional activity after rounds of restimulations.

In conclusion, owing to algorithm predictions (Table II - 1) and HLA class I and II typing, as previously reported in Table II - 2 (PCa-192: HLA-A*0101/0301, HLA-B*0701/1301, HLA-DRB1*0101/0401; PCa-196: HLA-A*0201/0201, HLA-B*0801/5101, HLA-DRB1*0101/1101; PCa-223: HLA-A*0201/1101, HLA-B* 4001/4401, HLA-DRB1*0701/1201; HD-GS: HLA-A*0201/6801, HLA-B*5101/5501, HLA-DRB1*0401/1401) we were also able to restrict the four peptides to specific HLA molecules: HLA-DRB1*0101/BK10 L-Tag$_{531-545}$, HLA-DRB1*1101/BK1 L-Tag$_{27-41}$, HLA-DRB1*0401/BK6 L-Tag$_{212-226}$ and HLA-A*0201/BK4 L-Tag$_{172-185}$.

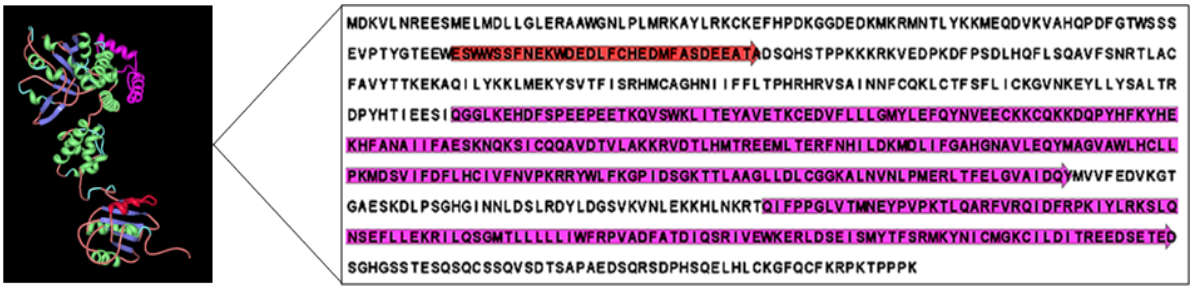


Figure II - 1. Structure and amino acid sequence of polyomavirus BK Large Tumor Antigen

The p53 binding domains are shown in magenta and the pRb binding region is highlighted in red.
(3D immage adapted from (Lilyestrom, Klein et al. 2006) PDB ID: 2H1L). (NCBI reference sequence: YP_717940.1)

Position	Sequence	HLA class II/I specificities (i)
BK1 L-Tag$_{27-41}$	**27-LPLMRKAYLRKCKEF-41** LPLMRKAYL LMRKAYLRK AYLRKCKEF	**DRB1*0101; *1101; *1501** B*07; B*08; B*53 A*03; A*11; A*30; A*31 A*23; A*24
BK2 L-Tag$_{69-85}$	**69-AHQPDFGTWSSSEVPTY-85** AHQPDFGTW FGTWSSSEV WSSSEVPTY	**DRB1*0101; *0901** A*24; A*23 A*02; A*68 A*01; A*26; A*29; B*15; B*35; B*58
BK3 L-Tag$_{148-164}$	**148-LSQAVFSNRTLACFAVY-164** LSQAVFSNR AVFSNRTLA FSNRTLACF RTLACFAVY TLACFAVY	**DRB1*0101; *0301; *0401; *0701; *1501** A*11; A*31; A*33; A*68 A*02; A*30; A*68 A*26; B*15; B*58; A*01; A*03; A*11; A*30; A*31; A*32; A*80; B*15; B*57 A*02
BK 4 L-Tag$_{172-185}$	**172-ILYKKLMEKYSVTF-185** ILYKKLMEK LYKKLMEKY KLMEKYSVT LMEKYSVTF	**DRB1*0101; *1101** A*03; A*11; A*30; A*23 A*24; A*80 A*02; B*15 A*32; B*15; B*35
BK5 L-Tag$_{196-209}$	**196-IIFFLTPHRHRVSA-209** IIFFLTPHR FLTPHRHRV TPHRHRVSA	**DRB1*0101; *0401; *0701; *0802; *1101; *1501** A*03;A*11; A*31; A*33; A*68 A*02; A*68; A*69; B*08 B*07; B*08; B*54
BK6 L-Tag$_{212-226}$	**209-NFCQKLCTFSFLICK-226** NFCQKLCTF QKLCTFSF KLCTFSFLI CTFSFLICK	**DRB1*0101; *0401; *0701; *1101; *1501** A*24 B*15 A*02; A*30; A*32 A*03; A*11; A*30; A*31; A*68
BK7 L-Tag$_{398-414}$	**398-CLLPKMDSVIFDFLHCI-414** CLLPKMDSVIF LPKMDSVIF KMDSVIFDF VIFDFLHCI	**DRB1*0101; *13012** A*02 B*07; B*15; B*35; B*53 A*32; B*15; B*58 A*02; A*68; A*69
BK8 L-Tag$_{410-426}$	**410-FLHCIVFNVPKRRYWLF-426** FLHCIVFNV HCIVFNVPK IVFNVPKRR VPKRRYWLF	**DRB1*0101; *0301; *0701; *0901; *1101; *1302; *1501** A*02; A*68; A*69 A*30 A*03; A*11; A*31; A*33; A*68 A*23; A*24; B*08
BK9 L-Tag$_{521-537}$	**521-QIFPPGLVTMNEYPVPK-537** IFPPGLVTM LVTMNEYPV TMNEYPVPK	**DRB1*0101; *0901; *1302** A*24 A*02; A*68 A*03; A*11; A*30; A*31; A*68
BK10 L-Tag$_{531-545}$	**531-NEYPVPKTLQARFVR-545** NEYPVPKTL YPVPKTLQA VPKTLQARF	**DRB1*0101** B*18; B*39; B*40; B*44 B*35; B*54 A*11; A*30; A*31; A*68
BK11 L-Tag$_{570-587}$	**570-ILQSGMTLLLLLIWFRPV-587** ILQSGMTLL LQSGMTLLL GMTLLLLLI MTLLLLLIW LLLLLIWFR LLLIWFRPV	**DRB*0101; *0701; *1501** A*02 A*02; B*15; B*39 A*02 A*24; B*15; B*53; B*57; B*58 A*11; A*31; A*32; A*33; A*68 A*02; A*69; B*08

Table II - 1. Candidate immunodominant peptides within L-Tag and their HLA class I/II associations
(i) HLA restriction has been assigned according to the predicted affinity score (IEDB for class I and NetMHCII for class II) given in nM IC50 values.

PCa patient code	HLA-A*	HLA-B*	HLA-DRB1*	BKV L-Tag serology (i)	BKV L-Tag viruria (ii)	BKV L-Tag viremia (ii)
PCa-2	* 0101/0201	* 4805/5504	* 1301/1301	++	-	-
PCa-8	* 2402/2501	* 0801/5501	* 0301/1601	++	-	-
PCa-9	* 0101/0301	* 4406/5701	* 0401/0701	++	+	+
PCa-10	* 0201/3201	* 2701/4402	* 0301/1301	+	-	-
PCa 11	* 0301/3201	* 0713/4002	* 1501/1501	-	-	-
PCa-13	* 0201/2501	* 5601/5701	* 0101/0701	+	-	-
PCa-62	* 0201/2408	* 0701/3525	* 0701/1303	++	-	-
PCa-63	* 0201/0201	* 1524/4402	* 1501/0401	+	-	-
PCa-68	* 0201/0301	* 4001/5601	* 0101/1301	++	-	-
PCa-69	* 0201/0212	* 0801/4402	* ?	+	-	-
PCa-70	* 0101/0201	* 0801/4402	* 0301/0701	+	-	-
PCa-71	* 0201/1101	* 3901/5303	* 1103/1405	-	-	-
PCa-73	* 0301/2402	* 4007/5514	* 1102/1501	++	-	-
PCa-74	* 0301/0301	* 0801/5504	* 0301/0301	+	-	-
PCa-75	* 1117/3301	* 0705/5301	* 0701/1102	-	-	-
PCa-76	* 1101/2901	* 4402/4402	* 1101/1301	+	-	-
PCa-192	* 0101/0301	* 0701/1301	* 0101/0401	+	-	-
PCa-196	* 0201/0201	* 0801/5101	* 0101/1101	++	-	-
PCa-223	* 0201/1101	* 4001/4401	* 0701/1201	+	-	-
BPH code	**HLA-A***	**HLA-B***	**HLA-DRB1***	**BKV L-Tag serology (i)**	**BKV L-Tag viruria (ii)**	**BKV L-Tag viremia (ii)**
BPH-1	* 0201/3010	* 1501/4001	* 1301/1301	++	-	-
BPH-2	* 0201/0301	* 4001/4601	* 0101/0301	++	-	-
BPH-3	* 0201/0201	n.d	* 1401/0820	+	-	-
BPH-4	* 0201/2402	* 4402/4402	* 0401/1501	++	-	-
BPH-5	* 0101/0101	* 4410/4410	* 0801/1101	++	-	-
BPH-6	* 0201/2901	* 1301/4001	* 0401/1501	++	-	-
BPH-7	* 0201/0201	* 4901/5101	* 1301/1501	-	-	-
BPH-8	* 1101/1101	* 4001/5501	* 0401/0825	+	-	-
BPH-9	* 2301/3101	* 1401/5701	* 0101/1101	+	-	-
BPH-10	* 0201/1101	* 0702/1501	* 1101/1101	+	-	-
HD code	**HLA-A***	**HLA-B***	**HLA-DRB1***	**BKV L-Tag serology (i)**	**BKV L-Tag viruria (ii)**	**BKV L-Tag viremia (ii)**
HD-TH	* 0201/3301	* 0801/5201	* 0301/1501	++	-	-
HD-LH	* 3204/3301	* 0702/4701	* 0701/1101	++	-	-
HD-GS	* 0201/6801	* 5101/5501	* 0401/1401	+	-	-
HD-MP	* 2402/3001	* 0702/0801	* 0301/1501	-	n.a	-
HD-EA	* 0201/0201	* 1301/5501	* 0401/1501	-	-	-
HD-1	* 1101/2402	* 4901/5101	* 1301/1401	+	n.a	-
HD-2	* 0101/0301	* 0701/5301	* 0101/1101	+	n.a	-
HD-3	* 0201/3201	* 2701/4202	* 0701/1501	+	n.a	-
HD-4	n.d	* 3901/3901	* 0401/1401	++	n.a	-
HD-5	* 2402/2402	* 5701/5701	* 0701/1201	+	n.a	-
HD-6	* 0201/0301	* 440201/3702	* 0101/1101	++	n.a	-
HD-7	* 2301/2301	* 0705/0705	* 0101/1101	-	n.a	-
HD-8	* 3101/0301	* 4001/4402	* 0401/0801	+	n.a	-

Table II - 2. HLA typing, BKV L-Tag serology, viruria and viremia of PCa patients, age- and gender-matched BPH patients and non-age-, gender-matched healthy donors

(i) IgG vs L-Tag (OD): (-) < 0.04; (+) < 0.1; (++) > 0.1

(ii) BKV L-Tag DNA molecular testing: (-) < 2 log copies/ml

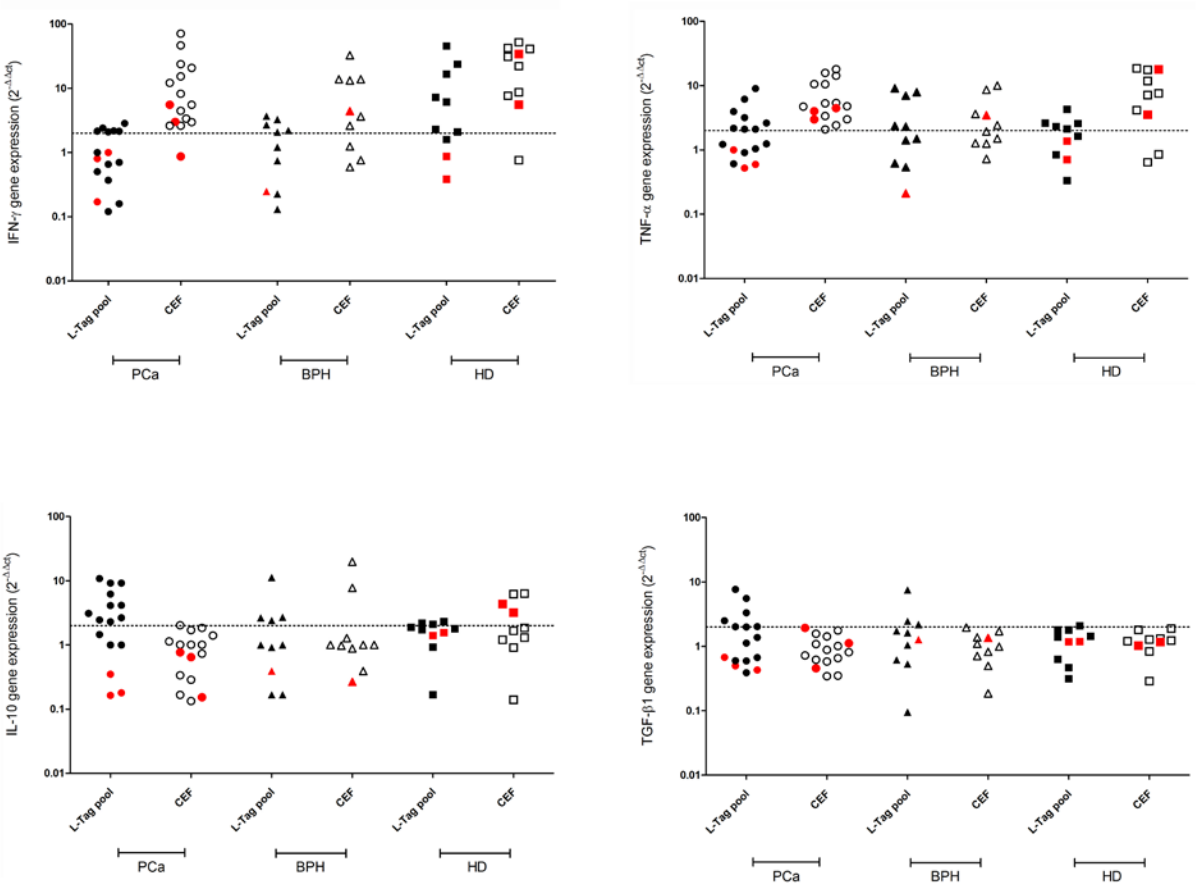


Figure II - 2. Cytokines gene expression profiling in PCa patients, BPH patients and HDs upon stimulation with a pool of overlapping peptides spanning the entire BKV L-Tag sequence

Ex vivo analysis of pro-inflammatory (IFN-γ and TNF-α) and immune regulatory (IL-10 and TGF-β1) cytokine patterns in PCa: n=16, BPH: n=10 and HDs: n=10 upon BKV L-Tag peptide-pool induction, as compared to a viral specific peptide-pool (CEF) induction. Peptide-induced cytokine genes expression was assessed after 4-hr incubation. Data are reported as cytokine gene relative quantification ($2^{-\Delta\Delta Ct}$). HIV peptide-pool was used as negative control to provide a baseline to compute fold changes. An arbitrary cut-off (dotted line) was set at 2-fold. BKV seronegative individuals are shown as red dots.

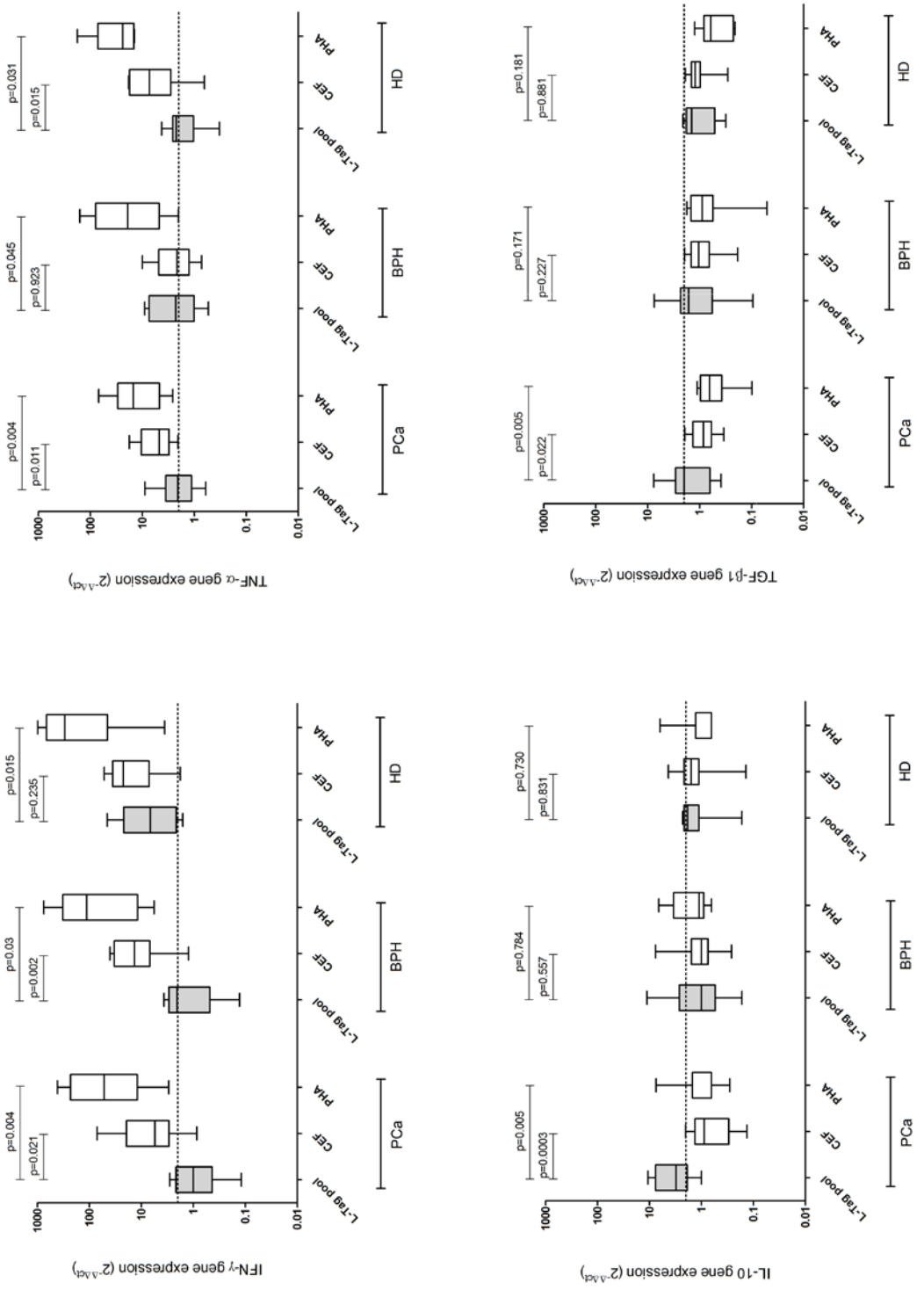


Figure II - 3. Cytokines gene expression profiling of BKV seropositive subjects upon stimulation with a pool of overlapping peptides spanning the entire BKV L-Tag sequence

Ex vivo analysis of pro-inflammatory (IFN-γ and TNF-α) and immune regulatory (IL-10 and TGF-β1) cytokine patterns in PCa: n=16, BPH: n=10 and HDs: n=10 upon BKV L-Tag peptide-pool induction, as compared to stimulation with a viral specific peptide-pool (CEF) and PHA. Peptide-induced expression of the indicated cytokine genes was assessed following 4-hr incubation. Data are reported as cytokine gene relative quantification ($2^{-\Delta\Delta Ct}$). HIV peptide-pool was used as negative control to provide a baseline to compute fold changes. An arbitrary cut-off (dotted line) was set at 2-fold.

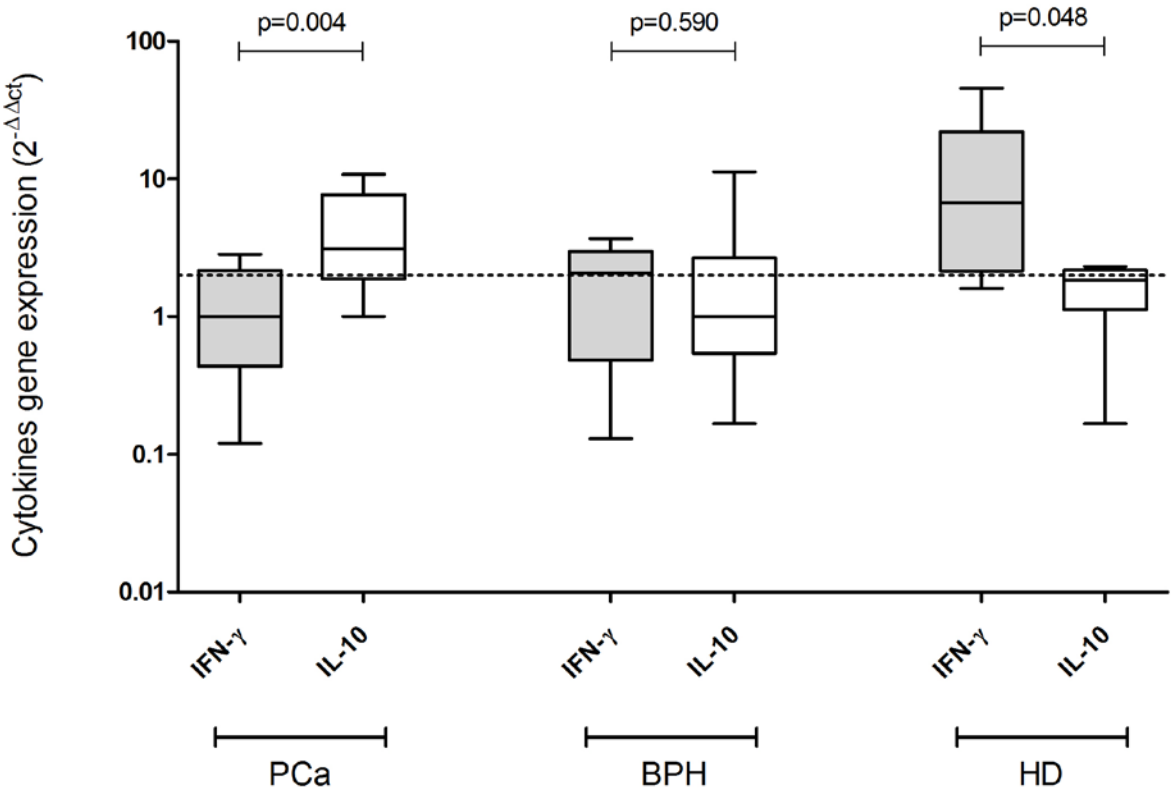
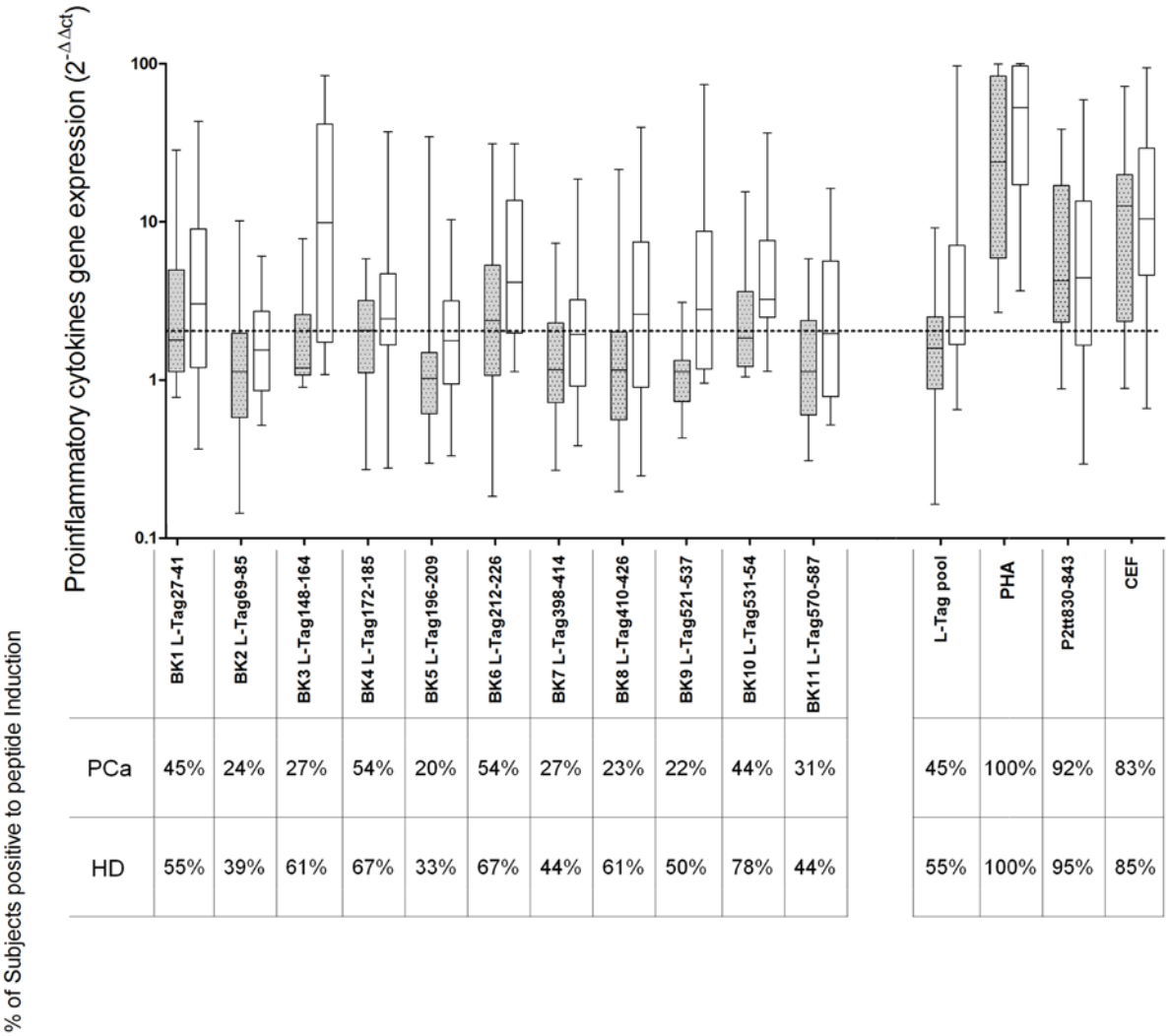


Figure II - 4. Differential pattern of immune responsiveness in BKV seropositive patients and donors triggered with a pool of overlapping peptides spanning the entire BKV L-Tag sequence

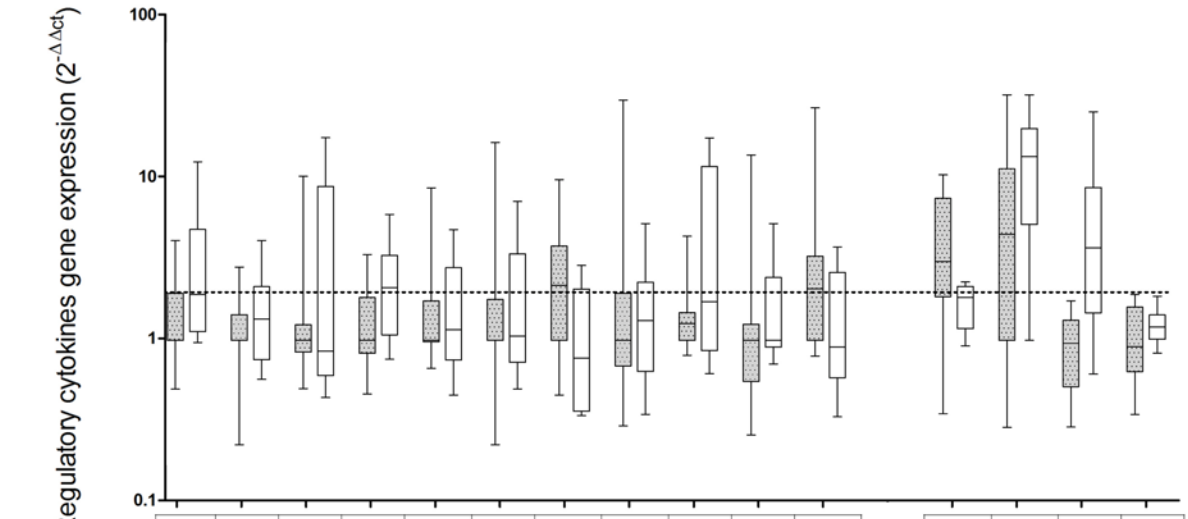
Ex vivo analysis of pro-inflammatory (IFN-γ and TNF-α) and immune regulatory (IL-10 and TGF-β1) cytokine patterns in PCa: n=16, BPH: n=10 and HDs: n=10 upon BKV L-Tag peptide-pool induction. Peptide-induced expression of the indicated cytokine genes was assessed following 4-hr incubation. Data are reported as cytokine gene relative quantification ($2^{-\Delta\Delta Ct}$). HIV peptide-pool was used as negative control to provide a baseline to compute fold changes. An arbitrary cut-off (dotted line) was set at 2-fold.



	BK1 L-Tag27-41	BK2 L-Tag69-85	BK3 L-Tag148-164	BK4 L-Tag172-185	BK5 L-Tag196-209	BK6 L-Tag212-226	BK7 L-Tag398-414	BK8 L-Tag410-426	BK9 L-Tag521-537	BK10 L-Tag531-54	BK11 L-Tag570-587	L-Tag pool	PHA	P2tt830-843	CEF
PCa	45%	24%	27%	54%	20%	54%	27%	23%	22%	44%	31%	45%	100%	92%	83%
HD	55%	39%	61%	67%	33%	67%	44%	61%	50%	78%	44%	55%	100%	95%	85%

Figure II - 5. Pro-inflammatory gene expression as detected in BKV seropositive PCa patients and healthy donors upon single BKV L-Tag peptides.

Analysis of pro-inflammatory (IFN-γ and TNF-α) cytokines gene expression in BKV seropositive PCa patients (n=16) and HDs (n=10) following *ex vivo* BKV L-Tag single peptides stimulation. Peptide-induced cytokines gene expression was assessed following 4-hr incubation. Three control peptides were used to compare mitogenic (PHA), viral-specific (CEF) and unspecific (P2tt830-843) antigenic stimulation. Data are reported as cytokine gene relative quantification ($2^{-\Delta\Delta Ct}$). HIV peptide-pool was used as negative control to provide a baseline to compute fold changes. An arbitrary cut-off (dotted line) was set at 2-fold. Interquartile min to max was reported for cytokines gene expressed upon induction. Only responses exceeding the cut-off (2-fold) were considered positive. (HDs: clear boxes; PCa patients: Dotted boxes).



% of Subjects positive to peptide Induction	BK1 L-Tag27-41	BK2 L-Tag69-85	BK3 L-Tag148-164	BK4 L-Tag172-185	BK5 L-Tag196-209	BK6 L-Tag212-226	BK7 L-Tag398-414	BK8 L-Tag410-426	BK9 L-Tag521-537	BK10 L-Tag531-54	BK11 L-Tag570-587	L-Tag pool	PHA	Tetanus	CEF
PCa	23%	15%	10%	20%	20%	12%	61%	23%	10%	10%	54%	85%	62%	1%	1%
HD	50%	25%	37%	50%	33%	37%	25%	37%	50%	37%	25%	37%	80%	70%	1%

Figure II - 6. Regulatory gene expression as detected in BKV seropositive PCa patients and healthy donors upon single BKV L-Tag peptides.

Analysis of regulatory (IL-10 and TGF-β1) cytokines gene expression in BKV seropositive PCa patients (n=16) and HDs (n=10) following *ex vivo* BKV L-Tag single peptides stimulation. Peptide-induced cytokines gene expression was assessed following 4-hr incubation. Three control peptides were used to compare mitogenic (PHA), viral-specific (CEF) and unspecific (P2tt830-843) antigenic stimulation. Data are reported as cytokine gene relative quantification ($2^{-\Delta\Delta Ct}$). HIV peptide-pool was used as negative control to provide a baseline to compute fold changes. An arbitrary cut-off (dotted line) was set at 2-fold. Interquartile min to max was reported for cytokines gene expressed upon induction. Only responses exceeding the cut-off (2-fold) were considered positive. (HDs: clear boxes; PCa patients: Dotted boxes).

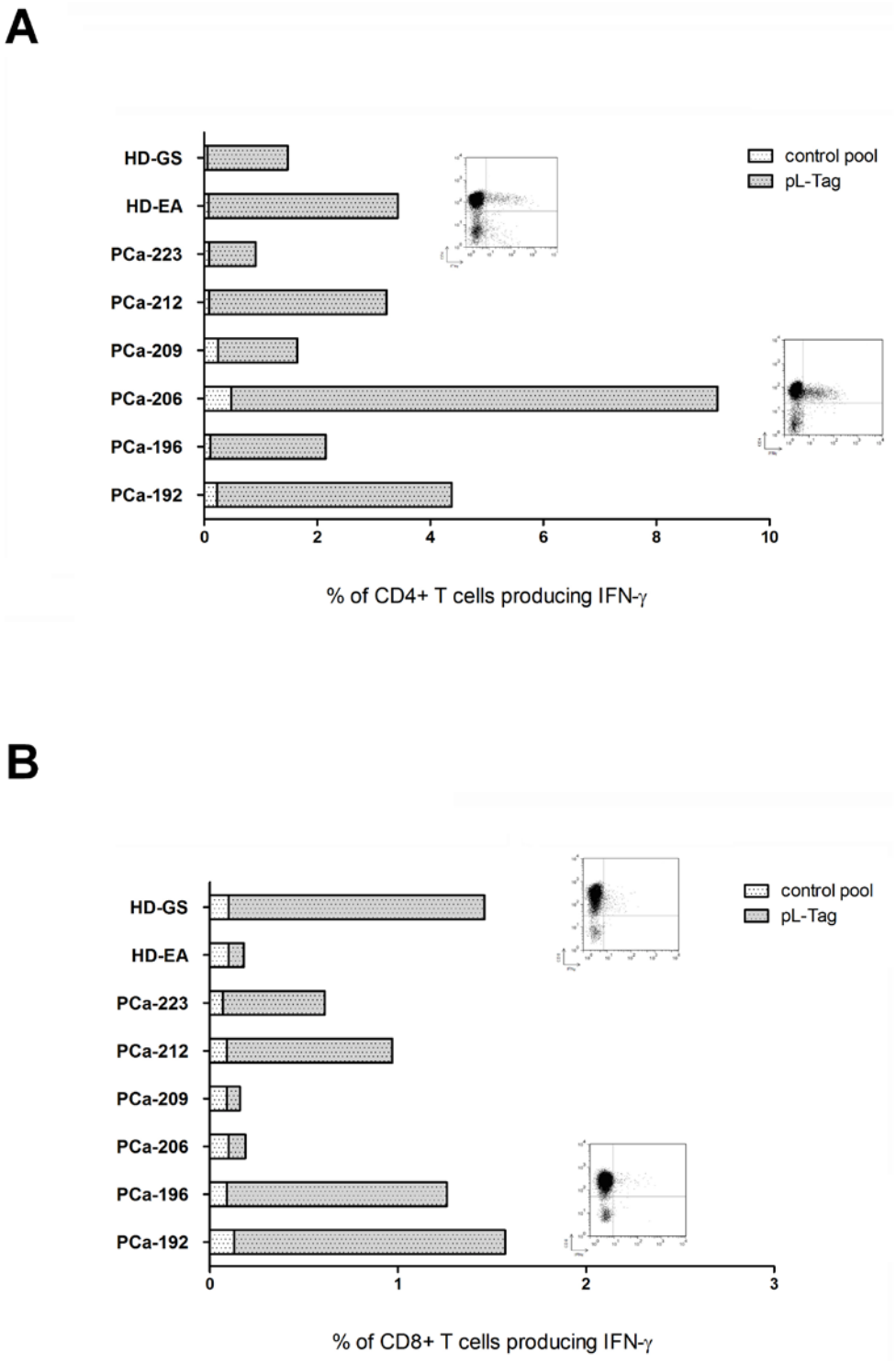


Figure II - 6. Immune responsiveness of CD4+ and CD8+ T cells triggered by pL-Tag
Purified CD4+ and CD8+ T cells were stimulated with autologous IFNDcs loaded with pL-Tag in the presence of IL-2. On day 14, cells were harvested and re-challenged using autologous IFNDCs pulsed either with pL-Tag or a control pool of irrelevant peptides (HIV). IFN-γ was measured in a standard ICS assay.

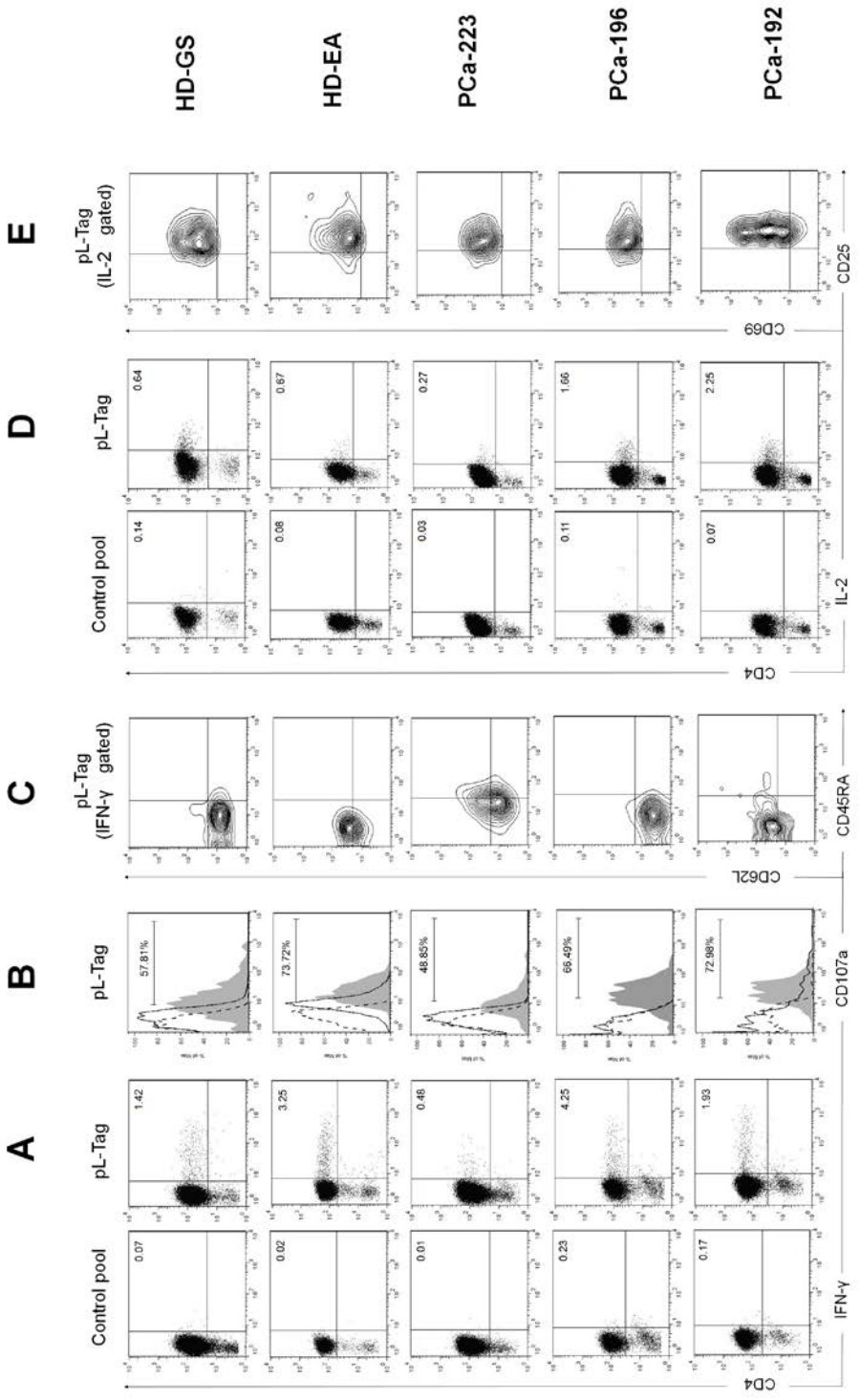


Figure II - 7 Functional and phenotypic characterization of pL-Tag induced CD4+ T cells

Representative ICS of pL-Tag induced IFN-γ (A) and IL-2 (D) production from five BKV seropositive individuals (three PCa patients and two HDs). Purified T cells were cultured for 2 weeks in presence of pL-Tag-pulsed autologous IFNDCs. On day 14, cells were harvested and re-challenged with either pL-Tag or a control pool of irrelevant peptides (HIV). Quadrants were set based on an isotype control. Data reported in the quadrants referred to percentages of CD4+ T cells showing evidence of cytokines production.

(B) CD107a expression for both IFN-γ producing (filled histogram) and IFN-γ negative (open histogram) CD4+ T cells was analyzed. The percentage of positive cells was indicated in each plot based on isotype control (dotted histogram). (C; E) Cytokines producing CD4+ T cells were stained either with CD45RA and CD62L (IFN-γ+ CD4+ T cells) or CD25 and CD69 (IL-2+ CD4+ T cells).

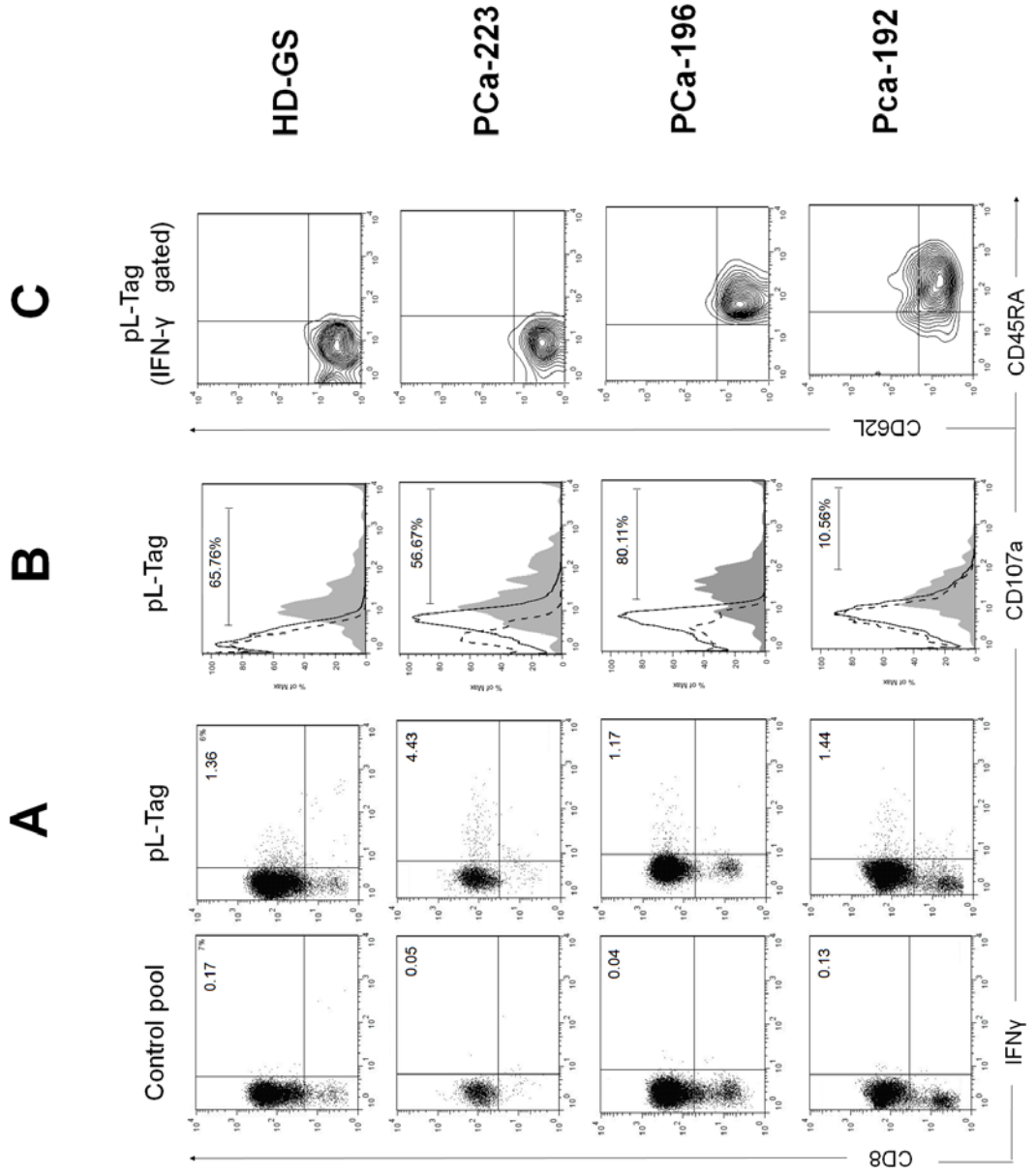


Figure II - 8 Functional and phenotypic characterization of pL-Tag induced CD8+ T cells
Representative ICS of pL-Tag induced IFN-γ (A) production from four BKV seropositive individuals (three PCa patients and one HD). Purified T cells were cultured for 2 weeks in presence of pL-Tag-pulsed autologous IFNDCs. On day 14, cells were harvested and re-challenged with either pL-Tag or a control pool of irrelevant peptides (HIV). Quadrants were set based on an isotype control. Data reported in the quadrants referred to percentages of CD8+ T cells showing evidence of cytokines production.
(B) CD107a expression for both IFN-γ producing (filled histogram) and IFN-γ negative (open histogram) CD8+ T cells was analyzed. The percentage of positive cells was indicated in each plot based on isotype control (dotted histogram). (C) IFN-γ producing CD8+ T cells were stained with CD45RA and CD62L.

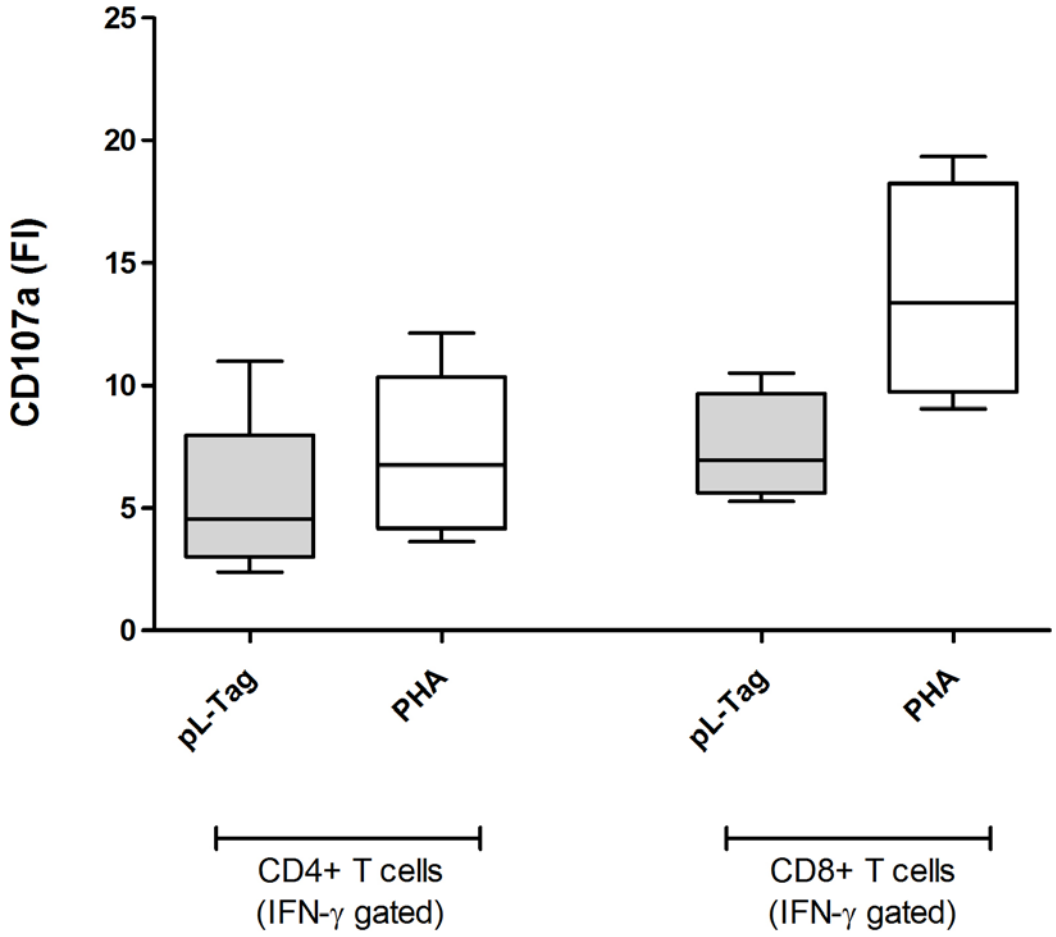


Figure II - 9 Analysis of CD107a expression in pL-Tag induced IFN-γ+ T cells
Comparative MFI analysis of CD107a expression among IFN-γ+ CD4+ and IFN-γ+ CD8+ T cells upon pL-Tag, PHA and irrelevant peptides (HIV) stimulation. Data were reported as fluorescence index (FI) calculated by the following formula: (MFI experimental sample – MFI background) / (MFI background) %. Boxes represent FI of CD107a staining among CD4+ and CD8+ T cells of four and three PCa patients, respectively.

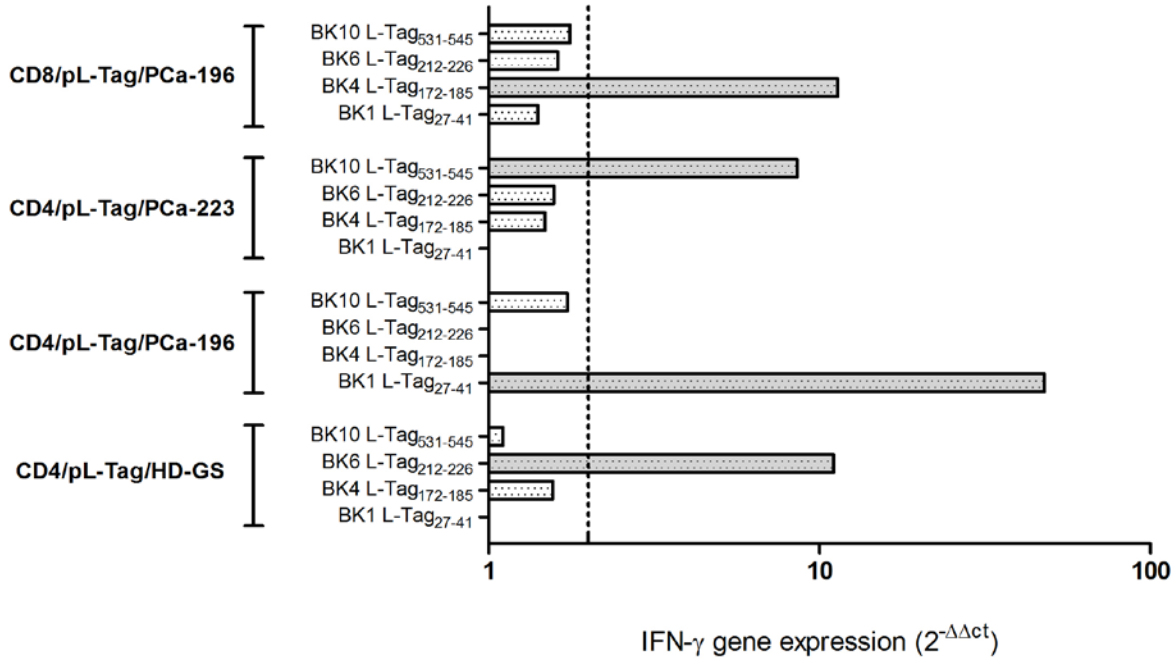


Figure II - 10 Cognate peptide recognitions within CD4+ and CD8+ T cell cultures
BK1 L-Tag$_{27-41}$, BK4 L-Tag$_{172-185}$, BK6 L-Tag$_{212-226}$ and BK10 L-Tag$_{531-545}$ specific T cells reactivation after pL-Tag *in vitro* sensitization of 3 PCa and 1 HD are represented by dotted gray histograms. Data were reported as IFN-γ gene relative expression in peptide triggered cells as compared to baseline generated by HIV peptides-pool stimulated counterparts. An arbitrary cut-off (dotted line) was set at 2-fold.

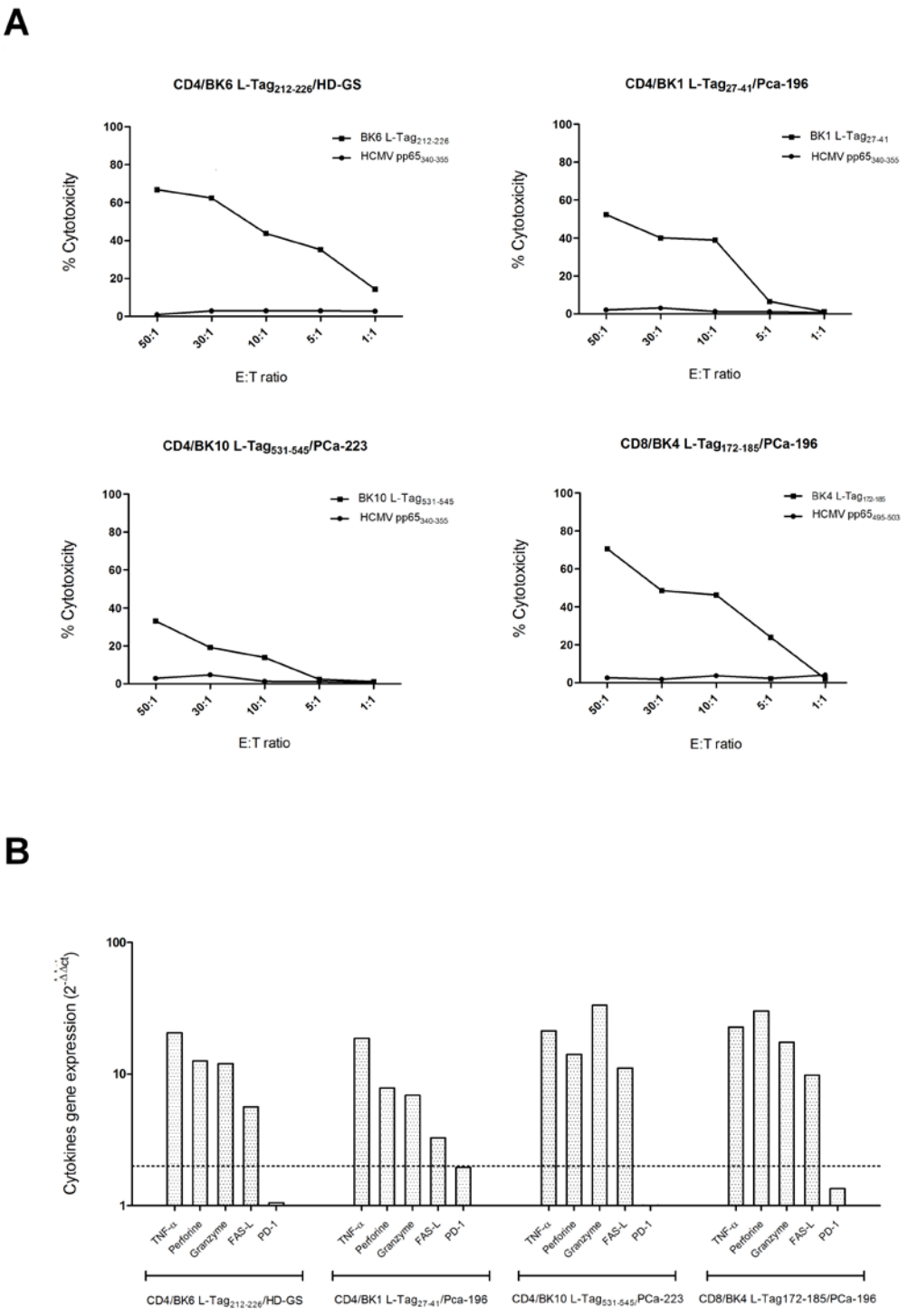


Figure II - 11 L-Tag peptides-specific T-cell polyclones recognize peptide-pulsed autologous dendritic cells and exert cytotoxicity

L-Tag-specific T-cell polyclones (three CD4+ and one CD8+) from PCa-196, PCa-226 and HD-GS were incubated with autologous mature dendritic cells (IFNDCs) pulsed with either respective specific peptides or an irrelevant peptide (HCMV $pp65_{340-355}$ for CD4+ T-cell polyclones and HCMV $pp65_{495-503}$ CD8+ T-cell polyclone).
(A) Cytotoxicity was measured in a LDH release assay. One representative experiment of two is shown.
(B) Expression analysis of genes encoding cytolytic effector molecules in L-Tag-specific T-cell polyclones. Data are reported as relative gene expression of peptide-specific T-cell polyclones as compared to stimulation with an irrelevant peptide (HCMV $pp65_{340-355}$ for CD4+ T-cell polyclones and HCMV $pp65_{495-503}$ CD8+ T-cell polyclone). An arbitrary cut-off (dotted line) was set at 2-fold.

5. Discussion

The evaluation and characterization of HLA-restricted epitope-specific memory T lymphocyte reactivity is an important step for the development of peptide-based immunotherapy for viral and tumor diseases. Our previous data support the concept that the *ex vivo* reactivation, and concomitant *in vitro* expansion, of memory T lymphocytes using single peptides or peptide-pools derived from viral antigens responsible for humoral responsiveness in either patients or healthy individuals infected with viruses encoding the cognate antigens, can effectively generate peptide-specific cytotoxic T lymphocyte (CTL) exerting immune activities in same subjects (Provenzano, Mocellin et al. 2003). Particularly, our previous results also state that specific HLA-A*0201-restricted peptides from polyomavirus BK L-Tag can efficiently be used to test immune responses in either HLA-A*0201+ BKV seropositive prostate cancer patients and HLA-A*0201+ non-age, gender-matched controls (Provenzano, Bracci et al. 2006; Sais, Wyler et al., 2009).

Despite bioinformatics-based methodologies for epitope mapping are less effective than approaches that use overlapping peptide-pools, the employment of several algorithms taking into account different features, such as peptide cleavage and MHC binding affinities, would overcome the problem of identifying immunogenic peptides across a wide range of MHC specificity. Indeed, in our experience, correlations between *in silico* predictions and peptide-induced immune responses, as analyzed by cytokine gene expression and cytotoxic T cell activity, ensures the identification of a substantial number of naturally processed antigenic peptides beyond HLA specificities (Provenzano, Sais et al. 2009).

As first screening, we proposed here the selection of 15-20mer peptides within BKV L-Tag by combining *in silico* predictions and cytokine gene expression profiling (qRT-PCR) (Provenzano, Mocellin et al. 2002; Provenzano, Bracci et al. 2006). The simultaneous analysis of genes encoding proinflammatory (IFN-γ, TNF-α, IL2) and immune regulatory (IL10, IL4, TGF-ß) cytokine patterns is informative about the nature of cellular immune responses elicited by candidate immunogenic peptides (Provenzano, Bracci et al. 2006, Sais, Wyler et al., 2009). We thus tested the ability of L-Tag peptides to strongly elicit CD4+ and CD8+ responses in prostate cancer patients (PCa), irrespective of specific HLA associations, in order to define the level of immunogenicity of these sequences in pre-early and early stages of the disease. Our intent was to investigate quantitative and qualitative features of CD4+ and CD8+ T cells responses against BKV L-Tag, as differentially detectable in BKV seronegative/positive PCa patients, and to compare them to that of both

gender- and age-matched BPH patients and gender- but non-age-matched healthy donors (HDs). In addition, we also aimed to confirm the evidence that polyfunctional CD4+ T cells have a direct crucial role in the control of viral infection (van de Berg, van Leeuwen et al. 2008; Zhou, Sharma et al. 2007).

We found that four out of the 11 peptides selected were able: i) to reactivate effector-memory CD4+ and CD8+ T cells from cancer patients showing an impaired pro-inflammatory cytokine (IFN-y and TNF-a) response upon L-Tag peptide pool stimulation, as reported before (Sais, Wyler et al. under review; Section 3.3); ii) to generate peptide specific CTL polyclones with functional features (degranulation of lytic machinery components and cytotoxic prolife) from single IFN-y secreting T cells, able in turn to exert a lytic activity on cognate peptide-loaded mature DC (mDC) in either BKV seropositive prostate cancer patients or healthy donors; iii) do not generate CTLs with an exhausted profile (PD-1) upon rounds of stimulations with BKV L-Tag, since chronic infection specifically up-regulates PD-1 in antigen-specific T cells (Day, Kaufmann et al. 2006) and its expression correlates with the functional impairment of those T cells (Barber, Wherry et al. 2006).

Considering both algorithm analysis and HLA typing carried out on accrued donors and patients, we also tempted to restrict HLA class I and/or class II alleles to the selected peptides. Despite the limited number of tests reported here, we were able to assign at least one HLA association to each peptide. However, further studies are needed to unquestionably assign HLA class I and II specific restrictions.

The analysis of BKV specific immune response in prostate cancer patients has required the use of multiple antigenic peptides. The large panel of specific peptides which encompass the entire antigen and that was under consideration in this study should have ensured a thorough evaluation of L-Tag specific responsiveness.

The future administration (systemic or local) of the four BKV L-Tag selected peptides using a co-stimulatory-molecules-enhanced recombinant Vaccinia Virus (rW) would ameliorate the BKV L-Tag T cell immune response in prostate cancer patients and increase the number of non-professional antigen presenting cells that could be identified as targets in the site of tumor growth (Adamina, Rosenthal et al. 2010). In addition, boosting BKV peptide-specific CTL responses might be envisaged either for the treatment of prostate cancer patients or as preventive procedure in individuals at risk of developing prostate cancer. The vaccination with rW encoding immunogenic peptides avoids the contrived administration of adjuvant to enhance peptide activity and to prevent peptide degradation by membrane and soluble enzymes expressed by antigen presenting cells microenvironment. (Cavazza, Adamina et al. 2004; Nagorsen, Servis et al. 2004)

The implementation of these studies might set the stage for original controlled investigations on mechanisms allowing BKV involvement in prostate cancers from active immune responses identifiable at early stages of prostate cancer and might provide sound scientific bases for the application of a wide range MHC peptide-vaccination in either preventive or therapeutic settings.

6. References

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7. Appendix

7.1. Table of Figures

7.1.1. Introduction

7.1.2. Chapters

7.1.2.1. Comprehensive characterization of BKV Large T antigen epitopes to promote the expansion of effectors T lymphocytes across a wide range of HLA class I and II antigens in prostate cancer

7.2. Curriculum Vitae

PERSONAL BACKGROUND

Name	**Giovanni SAIS**
Place / Date of birth	Lugano, Switzerland, 02.01.1983
Citizenship	Switzerland, Caslano / Ticino TI
Languages	Italian: mother tongue
	English: fluent
	French: very good knowledge
	German: very good knowledge

EDUCATION

2008 - present	**PhD student at the Department of Urology, Oncology and Tissue Engineering section, University Hospital Zürich, Switzerland**
	Faculty of Science, University of Zurich, Switzerland
	"*Generation of a Recombinant Vaccinia Virus encoding immunogenic BKV Large T antigen/p53 binding domains epitopes to promote the expansion of effector T lymphocytes across a wide range of MHC class I and II antigens in prostate cancer patients*"
	PhD Thesis committee: Prof. Anne Müller, Prof. Christoph Renner, Prof. David Nadal, Dr med Maurizio Provenzano
9.2005 - 12.2006	**Master of Science, Institute of Surgical Research and Hospital Management, Department of Clinical and Biological Science (DKBW), University Hospital Basel, Switzerland**
	Faculty of Science, University of Basel, Switzerland
	"*Comprehensive analysis of HLA class I and class II restricted epitopes from HCMV pp65 sequences"*
	Master Thesis committee: Prof. Giulio Spagnoli, Prof. Jean Pieters, Dr med Maurizio Provenzano

10.2002 - 12.2006	**Bachelor of Science in Molecular Biology** Faculty of Science, University of Basel, Switzerland
9.1998 - 7.2002	**Maturity diploma (scientific)** Liceo Cantonale Lugano I, Lugano, Switzerland

PROFESSIONAL EXPERIENCE

2.2011 – 4.2011	**Short-term Scholar, Thoracic Oncology Program, Cancer Center Hawaii (Prof. Michele Carbone)** University of Hawaii, Honolulu, USA
8.2007 - present	**Scientific researcher, Department of Urology, Oncology and Tissue Engineering section, University Hospital Zürich, Switzerland (Prof. Tulio Sulser)** University Hospital of Zürich, Zurich, Switzerland
9.2005 - 7.2007	**Scientific Researcher, Institute of Surgical Research and Hospital Management, Department of Clinical and Biological Science (DKBW) (Prof. Giulio C. Spagnoli)** University Hospital of Basel, Basel, Switzerland

PUBLICATION LIST

Peer-review articles

SAIS G, Wyler S, Banzola I, Mengus C, Bubendorf L, Hirsch Hans H, Sulser T, Spagnoli GC, Provenzano M (2011): L-Tag-specific systemic immune regulatory activity as a signature for polyomavirus BK involvement in prostate Cancer. Submitted to JVI

Provenzano M*, **SAIS G***, Bracci L, Egli A, Anselmi M, Viehl CT, Schaub S, Hirsch HH, Stroncek DF, Marincola FM, Spagnoli GC (2009): A HCMV pp65 polypeptide promotes the expansion of CD4+ and CD8+ T cells across a wide range of HLA specificities. J Cell Mol Med 2009 Aug;13(8B):2131-47. *equal contribution

Provenzano M, Bracci L, Wyler S, Hudolin T, **SAIS G**, Gosert R, Zajac P, Palu' G, Heberer M, Hirsch HH, Spagnoli GC (2006): Characterization of highly frequent epitope-specific CD4+5RA+/CCR7+/- T lymphocyte responses against p53-binding domains of the human polyomavirus BK large tumor antigen in HLA-A*0201+ BKV-seropositive donors. J Transl Med. 2006 Nov 10;4:47

Reviews

SAIS G, Spagnoli GC, Provenzano M (2007): Quantitative real time PCR: a rapid and sensitive screening method for T cell epitope-mapping. ASHI Quarterly 2007, 31(1): 12-13.

Provenzano M, Panelli MC, Mocellin S, Bracci L, **SAIS G**, Stroncek DF, Spagnoli GC, Marincola FM (2006): MHC-peptide specificity and T-cell epitope mapping: where immunotherapy starts. Trends Mol Med*.* 2006, 12(10):465-72

ATTENDED COURSES AND LECTURES DURING MY PHD

SS 2011	**Study design and data analysis using the statistical software R-1** Life Science Transferable Skills, University of Zürich, Switzerland (certified)
FS 2010	**Postgraduate course in improvement of scientific publication** Transferable Skills, University of Zürich, Switzerland (certified)
FS 2010	**Introduction to Management** University of Zürich, Switzerland (certified)
9.2009	**2nd Basel Immunology Focus Symposium, Immunological Tolerance, Department of Biomedicine Basel, Switzerland**
4.2010	**Writing a Scientific Paper for Publication Course, Institute of Clinical Research** University of Zürich, Switzerland (certified)
2.2009	**Introductory Course in Animal Science, University of Zurich, Switzerland** University of Zürich, Switzerland (certified)
8.2007 - present	**Seminar in Urological Research, University Hospital of Zürich, Switzerland**
8.2007 - present	**Colloquium, Institute of Surgical Research, University Hospital Zurich, Switzerland**
1.2008 - present	**Cutting Edge Topics: Immunology and Infection Biology, ETH, Switzerland**

2007 **Seminar in Genetic Approaches in Molecular and Biomedical Research, Biozentrum Basel, Switzerland**

SYMPOSIA CONTRIBUTIONS

Oral presentations

1.2011 **Colloquium Institute of Surgical Research, University Hospital Zurich, Switzerland**

"Comprehensive characterization of BKV Large T antigen epitopes to promote the expansion of effectors T lymphocytes across a wide range of HLA class I and II antigens in prostate cancer patients"

1.2011 **Seminar in Urological Research, University Hospital Zurich, Switzerland**

"Flow Cytometry Data Analysis with the use of Flowjo"

5.2010 **Seminar in Urological Research, University Hospital of Zürich, Switzerland**

"Flowcytomix: Multiple Analyte Detection"

10.2009 **Colloquium Institute of Surgical Research, University Hospital Zurich, Switzerland**

"L-Tag specific systemic immune regulatory profile as signature for polyomavirus BK involvement in prostate cancer"

4.2009 **The American Urological Association Annual Meeting (AUA), Chicago, USA**

"Impaired immune proinflammatory cytokine profiling in prostate cancer patients upon induction using peptides within polyomavirus BK-p53 binding regions"

9. 2008 **8th National Congress of Italian society of Virology, Orvieto, Italy**

"A HCMV polypeptide promotes the expansion of CD4+ and CD8+ T cells across a wide range of HLA specificities"

Poster presentations

6. 2009

BIT's 2nd Annual World Cancer Congress 2009, Beijing, China

"Impaired immune proinflammatory cytokine profiling in prostate cancer patients upon induction using peptides within polyomavirus BK-p53 binding regions"

4.2009

The American Urological Association Annual Meeting (AUA), Chicago, USA

"Impaired immune proinflammatory cytokine profiling in prostate cancer patients upon induction using peptides within polyomavirus BK-p53 binding regions"

J.Urol 2008 181 (4): 187 (Abstract)

2.2009

9th Charles Rodolphe Brupbacher Symposium, Zürich, Switzerland

"The Large T antigen-p53 binding domains exert an epitope an epitope specific immune regulatory profile in BK polyomavirus seropositive prostate cancer patients"

10.2008

18th European Society of Urological Research (ESUR) Meeting, Barcelona, Spain

"The Large T antigen-p53 binding domains exert an epitope an epitope specific immune regulatory profile in BK polyomavirus seropositive prostate cancer patients"

9.2007

10th European Society of Clinical Virology (ESCV) Meeting, Nurnberg, Germany

"Functional CD4+ and CD8+ T-cells expansion from potential human CMV seropositive transplant donors does not require defined MCH-peptide restrictions"

Awards and Grants

10.2008 **National Science Foundation (SNF) (co-applicant):**

"Generation of a Recombinant Vaccinia Virus encoding immunogenic BKV Large T antigen/p53 binding domains epitopes to promote the expansion of effector T Lymphocytes across a wide range of MHC class I and II antigens in prostate cancer patients"

10.2008 **Forschungskredit, University of Zürich, Switzerland:**

"Characterization of CTL immune activity against p53-binding regions of BKV large T antigen in BKV seropositive prostate cancer patients"

7.3. Acknowledgments

I would like to thank all the people who participated in the accomplishment of this work:

Prof. Dr. Tullio Sulser, for giving me the opportunity to perform my dissertation at the Division of Urology, under his direction.

PD. Dr. Maurizio Provenzano, my supervisor, who supported my work patiently offering an encouraging scientific education. My constant and sincere commitment to him during the course of my project was essential for my personal and scientific growth. He also gave me the opportunity to attend several national and international congresses. Maurizio was, and continues to be, not only an outstanding group leader, but also a confidant and a very good friend.

My scientific colleagues Irina Banzola and Damina Balmer for their true friendship, for always proving motivating spirit, for many cheerful moments and daily laughs we shared.

All the members of the Urological Research Group, University Hospital Zürich for their support and the pleasant working atmosphere.

The members of my “Promotionskomitee”: Prof. Anne Müller, Prof. Dr. Christoph Renner and Prof. Dr. David Nadal for their encouragement, feedbacks, suggestions and support.

Loredana for always trusting in my abilities, for her support and motivation, for listening to my problems and sorrows and for exploring together the non-scientific part of the life.

My family and friends, in particular my parents Giuliana and Sergio and my sister Anna-Chiara, for their endless love, endurance and support and for everything they gave me onto my way. I owe them everything.